American Journal of
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Reproductive Efficiency and Serological Findings in Heifers Experimentally Infected with *Vibrio Fetus*

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L. F. WILLIAMS, M.S.; E. M. KIGGINS, M.S.; E. WALKER, B.S.

Storrs, Connecticut

VIBRIOSIS has become recognized as the principal infectious cause of reduced reproductive efficiency in cattle. After the initial onset of the disease in a herd, undetected early abortions, apparent failure to conceive from three or more services, and off-cycle returns to estrus usually cause greater economic loss than detected abortions. It is generally agreed that the disease is self-limiting in a high percentage of females, that it is transmitted from bull to cow and from cow to bull during coitus, and that vibriosis may be transmitted by artificial insemination with untreated semen from infected bulls. A difference of opinion exists, however, as to whether transmission occurs by contact.

In experiments reported by Lawson and MacKinnon[1] and McEntee *et al.*,[2] spread of infection from artificially infected heifers to heifers used as controls was not observed. On the other hand, in an experiment reported by Plastridge *et al.*[3] and Easterbrooks and Plastridge,[4] infection demonstrated by isolation of the organism spread to 1, and on the basis of serological tests to a second, of 6 control heifers that were kept in an open barn and adjacent paddock in direct contact with 6 heifers inseminated with semen containing added live *Vibrio fetus* cells. Another control showed presumptive serological evidence of vibriosis.

The purpose of the present investigation was to obtain further information upon experimental transmission of vibriosis, and to secure data on the correlation of blood serum and cervicovaginal mucus agglutination reactions with breeding efficiency following cervical, vulval and, if occurring, contact exposure.

From the Department of Animal Diseases, Storrs Agricultural Experiment Station, University of Connecticut, Storrs.

Supported in part by funds provided by the Research and Marketing Act of 1946 for the regional research project NE1, Sterility in Dairy Cattle; and Lederle Laboratories Division, American Cyanamide Company, Pearl River, N. Y.

Dr. Easterbrooks is now with the Research Division, American Cyanamid Company, Pearl River, N. Y.

EXPERIMENTAL

Animals.—Ten dairy heifers from 6 to 8 months of age were placed in a barn, with adjacent paddock, located a half mile from other cattle. Blood serum and cervicovaginal mucus tests made prior to, and at the time of, exposure were negative in all dilutions. When the heifers were 14 to 16 months of age they were exposed to infection with *V. fetus* (strain R); 4 by swabbing the cervix with a suspension of viable *V. fetus* organisms and 4 by swabbing the vulva; 2 were left as controls to determine if they would acquire infection by association with the artificially exposed animals. The inoculum was prepared by suspending cells from a 48-hour-old culture in thiol medium, in tryptone broth to make a density of about 5.0 on the McFarland nephelometer scale.

All of the heifers were inseminated artificially with diluted semen from a single bull which was negative for *V. fetus* serologically and on repeated cultural examination. As an added precaution in eliminating the bull as a possible agent of transmission, 1,000 μg. of dihydrostreptomycin sulfate per milliliter were added to the diluted semen which was held in excess of four hours before use. Insemination of the heifers was started with the first heat which followed exposure. Periodic examinations for pregnancy were made starting forty-two days after the first insemination which was not followed by return to estrus. Breeding records were kept on each animal.

Serological Tests.—Blood and cervicovaginal mucus samples were collected and examined for *V. fetus* agglutinins at intervals of one to two weeks following exposure.

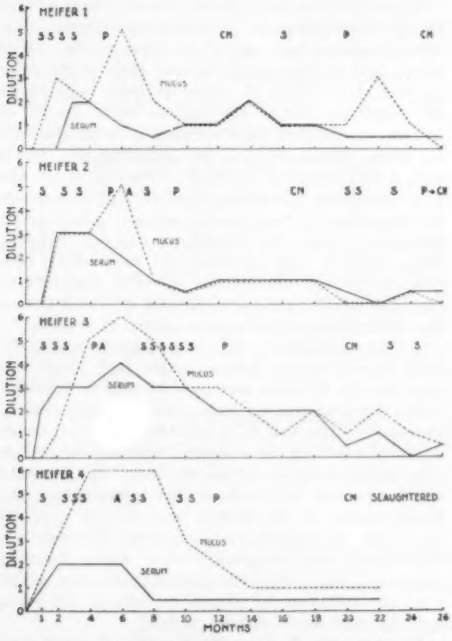
The agglutination antigen was made from *V. fetus* (strain R) by a procedure previously described by Plastridge *et al.*[3] with the exception that the suspending fluid consisted of Sorensen's phosphate buffer solution (pH 7.2) plus 0.3 per cent formalin. One liter of the solution contained:

$Na_2HPO_4 \cdot 2H_2O$.....8.313 Gm.
KH_2PO_4.....2.723 Gm.
Formalin (neutralized to pH 7.0 ± 0.2 by adding Li_2CO_3).....3.0 ml.

The antigen used in all tests agglutinated with known positive *V. fetus* antiserum, and failed to agglutinate with normal bovine serum in dilutions of 1:25 upward.

Blood serums were examined for *V. fetus* agglutinins by preparing twofold dilutions, starting with 1:25 and continuing through 1:1600, in 2-ml. amounts of the antigen and by observing the serum-antigen mixtures for evidence of agglutination after incubation at 37 C. for forty-eight hours and again after an additional twenty-four hours at room temperature.

Cervicovaginal mucus samples were collected by the tampon method and examined as described by Plastridge *et al.*[3,5] Concentrated antigen was added to 4 ml. of the mucus solution in tube 1 to make a density of 1.5 on the McFarland scale. Twofold dilutions were then prepared. The tubes were incubated and examined as in the tests on blood serum.


HEIFER 1
S S S S P CN S P CN
HEIFER 2
S S S P A S P CN S S S P+CN
HEIFER 3
S S S P A S S S S S P CN S S
HEIFER 4
S S S S A S S S P CN SLAUGHTERED
DILUTION
MUCUS
SERUM
MONTHS

Graph 1—Serological findings and breeding data following cervical exposure to Vibrio fetus.
S—service; P—pregnant; A—abortion; CN—calving normal.

RESULTS

The breeding records and results of serological tests following cervical, vulval, and contact exposure are summarized in figures 1, 2, and 3. In plotting the serological reactions after the first month, the average of the three weekly tests made at intervals of two months was used. The No. 1 serum dilution represents the 1:25 dilution and the No. 1 mucus dilution represents undiluted diluent-mucus mixture. Complete agglutination in the No. 1 tube in each test is referred to as a 1+ reaction and so on.

Cervical Exposure.—All of the 4 heifers exposed cervically (graph 1) became infected as evidenced by (1) either requiring more than three services, aborting after being diagnosed pregnant, or both, and (2) by the results of the serological tests. *Vibrio fetus* was recovered from the cervical mucus from 2 of the heifers.

Heifer 1 conceived from the fourth insemination and calved normally. Agglutinins appeared first in the cervicovaginal mucus, reaching the 3+ level in two months and the 5+ level in six months after exposure. The average serum reaction was 2+ three months after exposure, remained at this level for one month, and then gradually decreased to 1+ or less for the duration of the first breeding cycle. *Vibrio fetus* was recovered on cultural tests made three months after exposure. A tolerance for *V. fetus* had apparently developed by the time of the fourth insemination and recovery, as indicated by the serological tests, occurred between the sixth and eighth month following exposure.

The second conception and normal calving followed one insemination; the average serum titer remained low (1+ or less); the mucus titer showed a sharp temporary rise (3+) during the sixth month. The high mucus titer in the absence of clinical evidence of the disease suggests the possibility of a reinfection which the animal was able to resist.

Heifer 2 conceived from the third insemination, aborted during the third month of gestation, and required four inseminations for the first normal calving. Agglutinins appeared simultaneously in the serum and mucus, reaching 3+ two months following exposure. *Vibrio fetus*

was recovered on cultural tests made three months after exposure.

At the time of abortion, the average serum reaction was 2+ and the average mucus reaction 4+. Recovery apparently occurred shortly after she aborted.

After calving normally, seventeen months after exposure, she again conceived and calved normally.

Heifer 3 conceived from the third insemination, aborted about the second month of gestation, and required nine inseminations for the first normal gestation. The average serum reaction was 2+ one month after exposure, 3+ to 5+ during the period from two to ten months after exposure, and then decreased to 2+ or less. The average mucus reaction was 3+ to 5+ during the period from three to twelve months after exposure and varied between 2+ and 1+ thereafter.

At the time of abortion, the average serum reaction was 4+ and the average mucus reaction was 6+.

Recovery apparently occurred about the time of the conception that resulted in a normal calving.

Heifer 4 conceived from four inseminations, aborted between the sixth and ninth week of gestation, and required eight inseminations for her first normal calving. The average mucus reaction increased to 3+ two months after exposure and remained at or above this level for eight months and then decreased. The average serum reaction increased to 2+ two months after exposure, remained at this level for four months, and then decreased. Cultural tests made three, four, and eighteen months after exposure were negative for *V. fetus*.

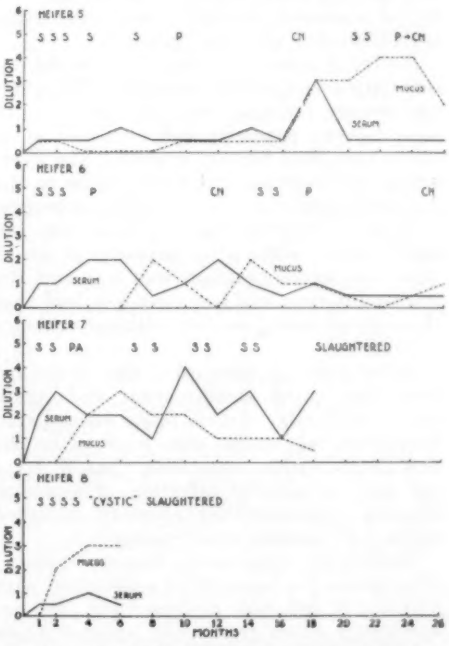
Both the breeding data and the mucus reactions indicate that recovery occurred about ten months after exposure and about the time that the animal conceived.

Vulval Exposure.—During the first breeding cycle following vulval exposure (graph 2), 2 (7 and 8) of 4 heifers showed clinical and serological evidence of vibriosis. One of these developed follicular cysts and was slaughtered six months after exposure.

Heifer 5 had an abnormally long (30 day) but regular estrous cycle. She was treated with an estrogen after the fourth insemination, conceived from the fifth insemination and calved normally. The average serum and mucus reactions remained low (usually less than 1+) during the first breeding cycle and cultural tests made on cervical mucus collected at three and four months after exposure were negative for *V. fetus*. The negative cultural and serological findings indicate that infection did not follow vulval exposure.

It is of special interest that heifer 5 became infected at, or near, the time of calving as shown by a sharp rise in serum and mucus titers, and the isolation of *V. fetus* two weeks after calving. It seems reasonable to assume that infection in this case was acquired from contact with infected animals.

Heifer 6 conceived after three inseminations and calved normally. The average serum reaction was 2+ four months after exposure, remained at this level for two months and then, with the exception of a 2+ reaction 12 months after exposure, remained under 2+ for the duration of the observation. The average mucus reaction was 2+ on two tests only. Cultural tests

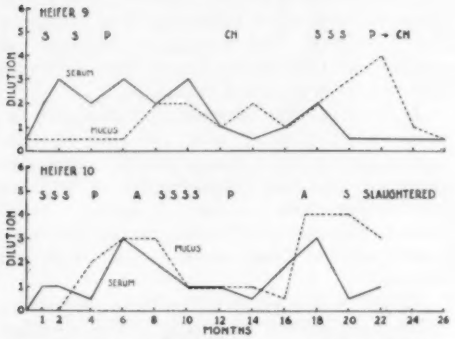


Graph 2—Serological findings and breeding data following vulval exposure to Vibrio fetus.

S—service; P—pregnant; A—abortion; CN—calving normal.

made three, four, and eighteen months after exposure were negative for *V. fetus*.

The results, as a whole, indicate that an infection failed to occur in this heifer following vulval exposure.


HEIFER 9
S S P CN S S S P → CN
SERUM
MUCUS
DILUTION
HEIFER 10
S S S P A S S S S P A S SLAUGHTERED
MUCUS
SERUM
DILUTION
1 2 4 6 8 10 12 14 16 18 20 22 24 26
MONTHS

Graph 3—Serological findings and breeding data following contact exposure to Vibrio fetus.

S—service; P—pregnant; A—abortion; CN—calving normal.

Contact Exposure.—Both of the 2 control heifers that were not artificially infected acquired infection from association with the other animals (graph 3).

Heifer 9 conceived from the second insemination and calved normally. The average serum reaction was 2+ one month after contact exposure and remained at or above this level for ten months. The average mucus reaction did not increase to the 2+ level until eight months after exposure.

It is of interest that *V. fetus* was isolated three months after exposure at which time the serum reaction was 3+ and the mucus reaction was less than 1+ and that this animal failed to show clinical evidence of vibriosis.

After calving normally, she conceived from the third insemination and again calved normally. *Vibrio fetus* was isolated from cervical mucus one week after the first insemination, indicating that *V. fetus* can exist as a latent infection. The mucus reaction indicates that recovery occurred about two months after conception.

Heifer 10 apparently became infected after about four months of association with artificially infected heifers. She was inseminated seven times and aborted twice during eighteen months. Both the average mucus and serum reactions were 3+ or higher at the time of the abortions, and 1+ for an interval of six months between the abortions. The second abortion occurred during the fifth month of gestation. It appears that either reinfection occurred or infection was latent for about six months following conception after the first abortion.

Cultural tests made three, four, and eighteen months after exposure and at the time of slaughter were negative for *V. fetus*.

Relation of Serological Reactions to Infection.—Findings obtained with the antigen and procedures used suggest the following interpretation of agglutination reactions for both the serum and mucus tests:

Tube 1*	Tube 2	Tube 3	Interpretation
−	−	−	Negative
i	−	−	
+	i	−	
+	+	i	Suspicious
+	+	+	Positive

* 1:25 dilution of serum, and undiluted diluent-mucus solution.

Grouping of the experimental animals during infection and during recovery is given in table 1. The "period of infection" was defined as the interval between the time of the first positive reaction to either test and the time both tests were negative or suspicious on three consecutive weekly tests. Negative mucus reactions obtained within three days of observed estrus were omitted. The "period of recovery" was defined as the interval between the time the serum and mucus reactions returned to negative or suspicious and the end of the period of observation, or the time either test was again positive on three consecutive weekly tests.

During infection, 315 serum and mucus tests were made. The results clearly indicate that both tests should be used in diagnosing vibriosis. When the results of both

TABLE 1—Grouping of Animals on the Basis of Serum and Mucus Tests During Infection and During Recovery

Reaction	During infection (315 tests)			During recovery (334 tests)		
	Serum test (%)	Mucus test (%)	Both tests (%)	Serum test (%)	Mucus test (%)	Both tests (%)
Negative	47.6	21.0	3.5	83.3	75.5	63.8
Suspicious	26.3	15.9	20.0	15.3	21.9	32.3
Positive	26.0	63.2	76.5	1.5	2.7	3.9

tests were used the animals were classed as negative 3.5 per cent of the time, suspicious 20.0 per cent of the time, and positive 76.5 per cent of the time. While the mucus test detected infection more often than the serum test, the serum test was frequently positive when the mucus test was negative.

During "recovery" 334 serum and mucus tests were made. The serum test was negative more often than the mucus test. This is to be expected, since the serum titer in the majority of the animals decreased more rapidly than the mucus titer. Nevertheless, when the results of both tests were used the recovered animals would have been classed as positive only 3.9 per cent of the time.

DISCUSSION

Cervical exposure to *V. fetus* resulted in infection in all of 4 heifers, as indicated by clinical evidence of vibriosis and the development of agglutinins in the blood serum and cervicovaginal mucus. Recovery, as indicated by the criteria used, occurred in all 4 heifers within eight to twelve months. The time required for the development of significant levels of agglutinins in either the serum or mucus was from one to two months following exposure. The duration of significant serum titers (suspicious or positive) was one, four, four, and seventeen months, respectively; the duration of significant mucus titers was five, six, ten, and twenty months, respectively. It is of interest that the agglutinin content of the cervicovaginal mucus of 3 of the 4 heifers dropped sharply to the suspicious or negative level in from one to two months following the insemination that resulted in a normal gestation but persisted for about five months in the fourth heifer.

Vulval exposure was followed by more variable results with 2 of 4 heifers becoming infected.

Contact exposure resulted in infection in 3 heifers. Both controls became infected within four months and 1 of the heifers exposed by the vulval route, but which remained negative to the serum and mucus tests, apparently became infected in sixteen months. *Vibrio fetus* was isolated from 1 of the 2 control heifers. While cultural tests on the other heifer were negative both the clinical and serological evidence showed that she became infected.

The finding that the production of *V. fetus* agglutinins in the blood serum and in the cervicovaginal mucus can occur independently presents an interesting field for speculation. It would appear that the presence of agglutinins in the cervicovaginal mucus and their absence in the blood is indicative of the presence of *V. fetus* on the cervical mucosa and adjacent vaginal mucosa without pathological changes. The presence of agglutinins in the blood and their absence in cervicovaginal mucus would appear to indicate localization of infection in the uterus. This may also explain the comparatively short duration of blood serum titers in some animals, since uterine infections tend to be of short duration, as indicated by cultural tests on several animals that were slaughtered following vibrionic abortion.[6] The simultaneous presence of agglutinins in the blood and mucus would seem to indicate either both uterine and cervical infection (with or without pathological changes) or cervicitis due to *V. fetus*. Under these conditions, disappearances of *V. fetus* from the uterus or healing of the cervical mucosa could result in the disappearance of agglutinins from the blood and the persistence of agglutinins on the cervical mucosa until the organisms disappeared from this site also.

In general, in this limited experiment, the presence of agglutinins in the cervicovaginal mucus was a more constant criterion of infection than the presence of agglutinins in the blood serum; however, at times agglutinins were present in the blood and absent in the mucus (heifer 9) and vice versa (heifer 8). This finding demonstrates that both blood serum and cervicovaginal mucus should be examined in any attempt to diagnose vibriosis accurately by serological means.

With the antigen used, it appears that a serum titer of less than 1:50 should be interpreted as negative, a titer of 1:50 as suspicious (positive if associated with abortion), and a titer of 1:100 or higher as positive. In testing cervicovaginal mucus for agglutinins, concentrated antigen was added to 4 ml. of the centrifuged mucus-diluent solution to make a concentration of about 1.5 on the McFarland scale. Twofold dilutions were then prepared. The results

indicate that agglutination in the tube (No. 1) containing the undiluted mucus solution only should be interpreted as negative, agglutination in tube No. 2 as suspicious, and agglutination in the No. 3 tube and beyond as positive.

Cultural tests on cervical mucus were not reliable in detecting infection and were usually negative unless made during the early stages of infection.

The results obtained on the heifers that aborted show that in diagnosing vibrionic abortion, both blood and cervicovaginal mucus samples should be taken within one week following abortion. Mucus samples should not be taken within four days of observed estrus. Agglutinins usually persisted in the mucus for eight weeks following an abortion from vibriosis but tended to disappear in most animals thereafter. In one of the six abortions that occurred in the experimental heifers, both blood and cervicovaginal mucus agglutinins disappeared within two weeks following abortion.

SUMMARY

Infection, as shown by clinical evidence (abortion and apparent failure to conceive from three, or more, services or both) and the presence of agglutinins in either the blood serum or cervicovaginal mucus or both, followed cervical exposure of 4 of 4 heifers, vulval exposure of 2 of 4 heifers, and contact exposure of 2 of 2 heifers plus 1 of the 4 heifers that failed to develop serological evidence of vibriosis following vulval exposure.

The interval required for the development of significant levels of agglutinins in the blood and cervicovaginal mucus following cervical exposure was four to twelve weeks.

Recovery, as indicated by conception from one to three services followed by a normal gestation and a decline in serum and cervicovaginal mucus titers, occurred within eight to fourteen months in 5 of 8 heifers that became infected and were retained on the experiment. Infection persisted in 1 heifer for the eighteen months of observation. Two heifers appeared to recover after ten to twelve months but later either became reinfected or latent infection became reactivated.

The appearance of agglutinins in the blood serum was independent of agglutinin formation in the cervicovaginal mucosa and vice versa. For this reason, both blood serum and cervicovaginal mucus should be tested for agglutinins in diagnosing vibriosis.

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The October, 1955, issue of the AMERICAN JOURNAL OF VETERINARY RESEARCH will be in two parts. This is Part 1.

Part 2 will be the bibliography of "Diseases in Wild Mammals and Birds" compiled by Dr. Patricia O'Connor Halloran, of the Staten Island Zoo, Staten Island, N. Y.

Physiological Studies of the Vagal Nerve Supply to the Bovine Stomach. II. Studies on the Eructation Mechanism in Adult Cattle

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THE importance of acute bloat has been well recognized. Most current investigations of bloat are in the direction of studying factors which may disturb the eructation of gas.[1-4]

In approaching the problem, it appeared that study of the eructation mechanism might be aided by using the chronic intrathoracic vagal electrode technique.[5]

The present study was undertaken in order to determine the applicability of this technique to adult cattle with rumen fistulas, and to determine if improvements in quantitation of motor events in the ruminant stomach could be effected.

METHODS

Surgical.—Thoracotomy was performed on 3 adult Holstein-Friesian cows, each of which had a rumen fistula, for the purpose of applying electrodes on the dorsal vagal trunk.[5]

The rumen and reticulum were emptied of ingesta prior to operation, and ¼ grain of atropine sulfate was administered subcutaneously to minimize collection of fluids in the pharynx. The animals were placed under general anesthesia with chloral hydrate, pentobarbital sodium, magnesium sulfate solution.[6]

Artificial respiration was provided by two intratracheal oxygen catheters inserted to the mid-cervical region of the trachea. The middle third of the left eighth rib was resected after reflecting the periosteum. The free ends of the electrode wires were brought out of the thorax and anchored in the subcutis.

Electrodes were similar in construction to those used in previous studies[5] except that larger polyethylene * housings were on the wire ends applied to the nerve. A continuous piece of 18-gauge silver wire,** insulated with polyethylene † tubing, was used; the tubing joints were filled with melted polyethylene plastic.

Studies of the responses to dorsal vagal stimulation ‡ were carried out at frequent intervals starting approximately two weeks postoperatively and continuing three to six months later. Prior to each experiment, the electrodes were routinely checked for breakage as follows:

(*a*) Ohmmeter readings were taken of electrode preparation; these varied from animal to animal, but were reasonably constant for a given animal, with time. The range encountered in the functional electrode preparations here reported was 300 to 6,000 ohms.

(*b*) Stomach compartments were palpated during trials of various stimuli.

(*c*) Radiograms proved effective in locating broken areas. The breaks were usually in the subcutis or muscle and not difficult to repair. The cows were kept in individual box stalls to prevent damage to the subcutaneous wire ends by other cows.

Manometric.—A four-channel electronic pressure-recording system was used for recording pressure changes in the cardia, reticulum, anterior dorsal rumen sac, and posterior dorsal rumen sac. The system employed unbonded strain-gauge transducers,§ an electromanometer, and a hot wire recorder,‖ with water-filled polyethylene tubes * as described by Brody and Quigley,[7-9] with whistle tips used as pressure receivers. Three of these tubes were placed in the reticulum, cardia, and anterior dorsal rumen sac through a nasal stomach tube which was then withdrawn. The fourth tube to the posterior dorsal sac was passed through one valve stem of a Dougherty[10] pneumatic rumen fistula plug. The first three tubes were anchored by ½-ounce lead weights in their respective compartments. The weight for the cardia receiver was placed in the reticulum with the end closed; the tube opening, about 12 inches from the end of the tube, was placed about 1 inch anterior to the rim of the funnel-shaped cardiac antrum.[11]

Using a Dougherty fistula plug, modified so as to seal around the observer's arm placed through a cut-out center portion, the respective compart-

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* PE 440, Clay-Adams Co., Inc., New York, N. Y.

** American Platinum Works, Newark, N. J.

† PE 200, Clay-Adams Co., Inc., New York, N. Y.

‡ Square wave electronic stimulator, Model 751, American Electronics Laboratories, Inc., Philadelphia, Pa.

§ P 23 A, Statham Laboratories, 254 Carpenter Rd., Hato Rey, Puerto Rico.

‖ The Sanborn Co., Cambridge, Mass.

* PE 280, Clay-Adams Co., Inc., New York, N. Y.

ments were palpated during contractions and during eructation, swallowing, and regurgitation, with a mirror permitting viewing of the recording styli. It was determined in this manner that in no case was a recording tube-tip recording pressure change in compartments other than its own, except as noted below; it was also determined that eructations, swallowings, and regurgitations were easily distinguished from each other on the tracing (graph 1 A). Also, the signal marker was used to record, independently, events in the esophagus, including eructation, swallowing, and regurgitation. These observations were made (*a*) visually and (*b*) audibly by the use of amplified esophageal sounds from a microphone ** held over the esophagus by a long chest-type electrocardiograph rubber tape.

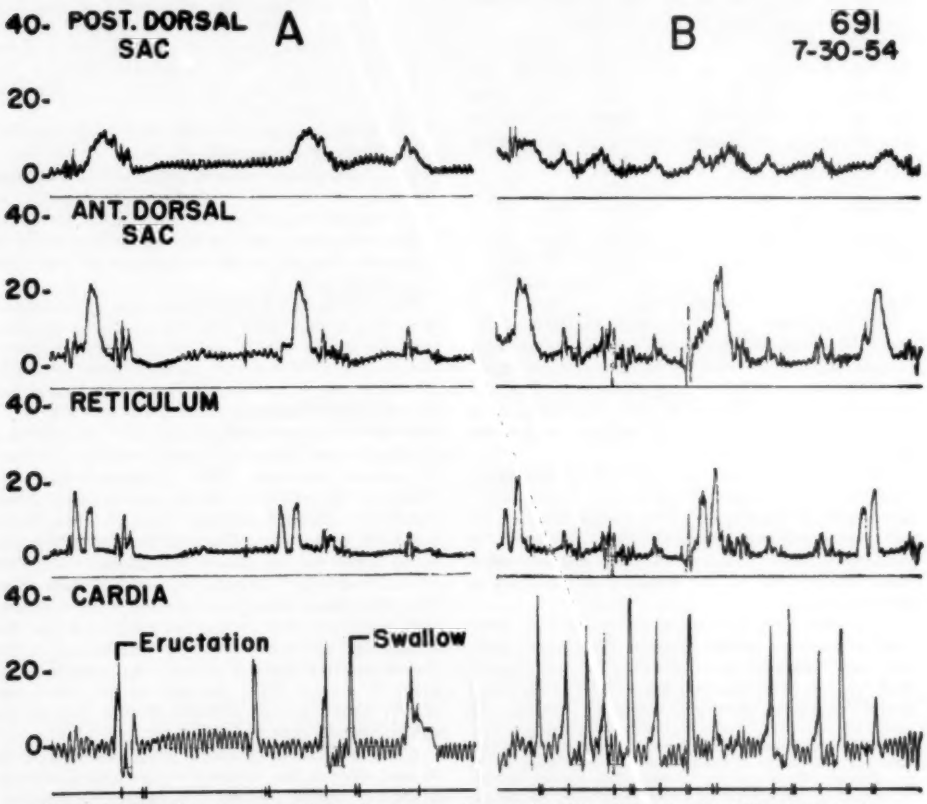
** Maico Stethetron, Minneapolis, Minn.

A Gilson mixing adapter † was used to convey a slow drip of water into the recording system from a reservoir bottle suspended 6 feet above the level of the transducer. The base line was not markedly affected by this slow drip, which served to keep the recording tips free of ingesta.

At the time of coughing or sudden somatic movement, deflections occurred in all tracings. Likewise (graph 1 A), when eructation occurred, it was likely to be reflected on all tracings. The regular motor events, in the several stomach compartments, including abomasum as well as those previously mentioned, were not recorded on any tracing other than the channel recording from the given compartment.

The system was calibrated statically with a mer-

† Becton-Dickinson Co., Rutherford, N. J.

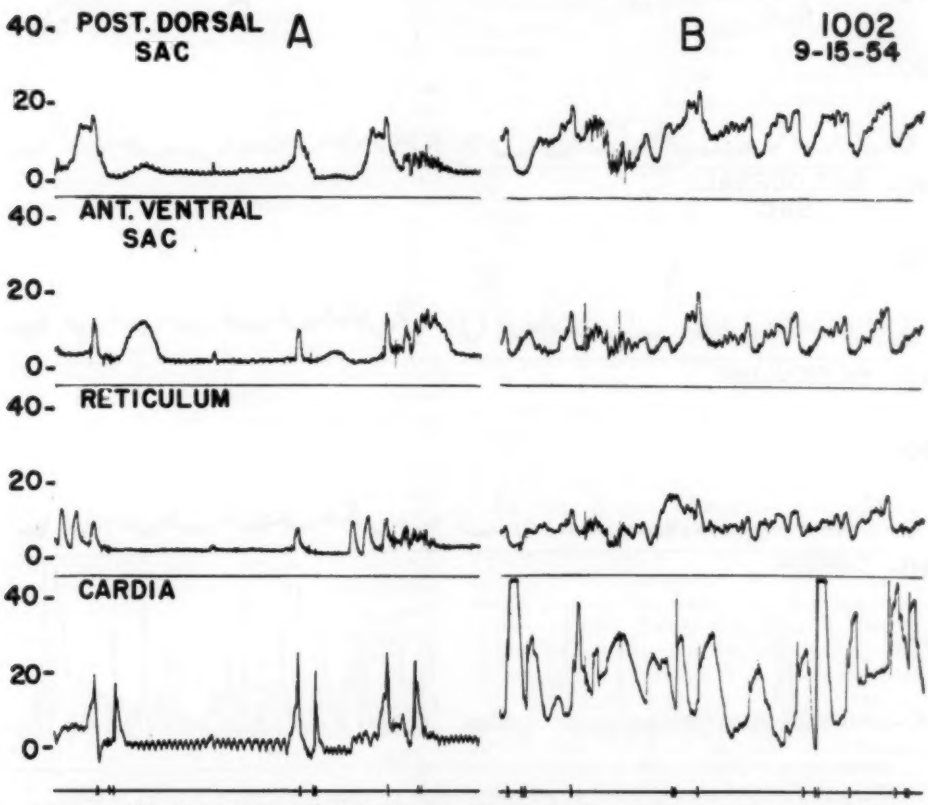


Graph 1—Simultaneous recordings of pressure changes (reading from top to bottom) in posterior dorsal rumen sac (gas pocket), anterior dorsal rumen sac (floor), reticulum (floor), and antrum of the cardia. Record A shows spontaneous motor events. Record B, taken immediately after record A, shows effect of moderate electrical stimulation, in the unanesthetized state, of the dorsal vagal trunk. Air insufflation rate, records A and B, was 5 liters per minute. Stimulus used: 10 cycles per second, 5 milliseconds pulse duration, 10 volts. Horizontal axis, 1 large division = 5 seconds; vertical axis, 1 large division = 5 mm. of mercury pressure.

cury manometer before and during experiments. Dynamic calibration using the criteria and square wave method (using balloons) of Wood,[12] and suggestions of Code *et al.*,[13] showed the system to have a natural frequency of approximately 7 cycles per second, a lag of approximately 0.04 seconds, with approximately a 10 per cent overshoot, when nearly critically damped. This system consisted of two lengths (10 ft. each) of polyethylene tubing ‡ joined by adapters * and the side tube and reservoir with a screw clamp placed immediately adjacent to the mixing adapter. In practice, the screw clamp was moved several inches up the side tube in order to overdamp the system somewhat, since the critically damped system, at the amplification used, was too sensitive to slight movements on the part of the animal for clear inscription of the fundamental wave forms. Respiratory waves are superimposed on the tracings (graph 1 A) even when overdamped as described. Further studies are in progress in an effort to refine further the quantitative aspects of the tracings, but the above system seemed adequate for the present studies.

Skin areas opposite the recording tips were located by palpation from within (*i.e.*, elevating the wall) and manometers were leveled with these points, thus recording depths of ingesta over the recording tips, in the initial part of the tracing. The styli were then adjusted to the arbitrary base line (graph 1 A) for recording subsequent pressure changes.

‡ PE 280, Clay-Adams Co., Inc., New York, N. Y.
* Becton-Dickinson Co., Rutherford, N. J., Luer-lok, 1/16 in. I.D. tubing adapters.



Graph 2—Simultaneous recordings of pressure changes (reading from top to bottom) in the posterior dorsal rumen sac (gas pocket), anterior ventral rumen sac (floor), reticulum (floor), and antrum of the cardia. Record A shows spontaneous motor events. Record B, taken immediately after record A, shows effect of strong electrical stimulation, in the unanesthetized state, of the dorsal vagal trunk. Notice spasmodic contractions of rumen and cardia. Air insufflation rate, records A and B, was 5 liters per minute.
Stimulus used: 10 cycles per second, 5 milliseconds pulse duration, 30 volts. Horizontal axis, 1 large division = 5 seconds; vertical axis, 1 large division = 5 mm. of mercury pressure.

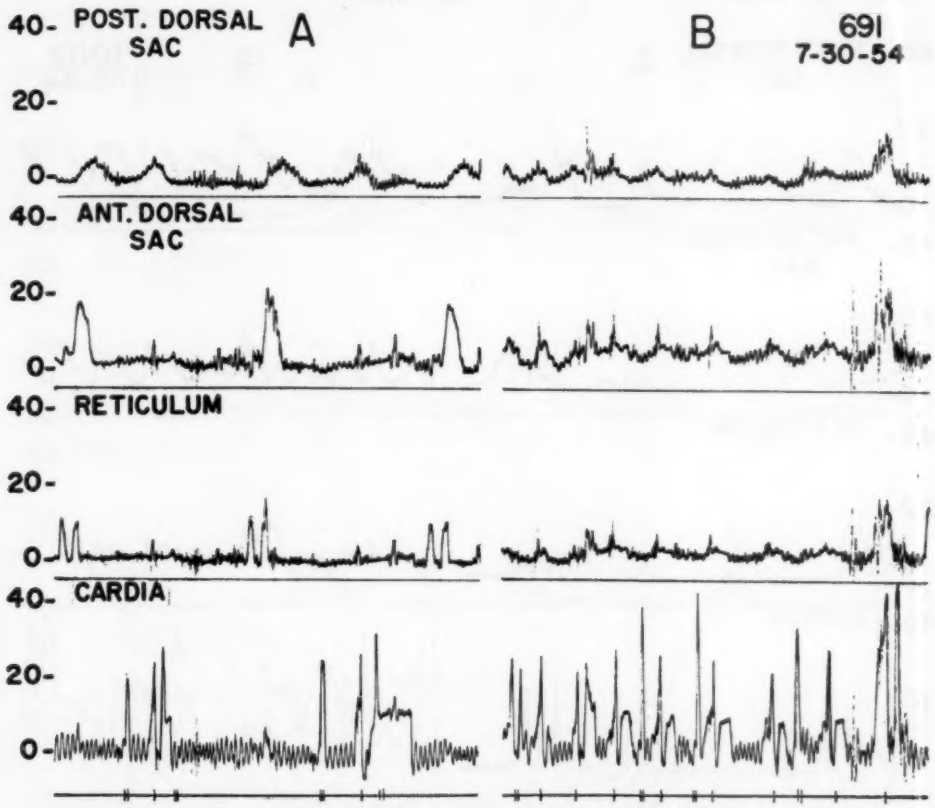
Graphs 1 to 3 are photographs of records characteristic of the results obtained on the 3 cows. Each record represents a total of four minutes of recording (2 minutes, record A; 2 minutes, record B). Each large division on the vertical axis is equivalent to 5 mm. of mercury pressure. Each large division on the horizontal axis is equivalent to five seconds.

RESULTS

SURGICAL

The animals made complete recoveries. They were used, starting about two weeks following thoracotomy, from once to several times a week for the period over which the electrode preparations were functional. This period varied from three months to approximately six months. One animal, still operative, has been used about seven months.

For each use, a small (1 in.) skin incision was made, after procaine, to permit the withdrawal of the wire ends for stimulation. After use, the wire ends were bent and tucked subcutaneously, crystalline penicillin instilled, and a single silk suture used to close the incision. These incisions repeatedly healed in good order but tended, after a time, to begin suppu-



Graph 3—Simultaneous recordings of pressure changes (reading from top to bottom) in posterior dorsal rumen sac (gas pocket), anterior dorsal rumen sac (floor), reticulum (floor), and antrum of the cardia. Record A shows spontaneous motor events, including regular eructations, with hindquarters elevated and cardia submerged 6 to 8 inches with water. Record B, taken immediately after Record A, shows the effect of moderate electrical stimulation, in the unanesthetized state, of the dorsal vagal trunk under these conditions. Notice increased eructation and swallowing rates in record B. Air insufflation rate, records A and B, was 5 liters per minute. Stimulus used: 10 cycles per second, 5 milliseconds pulse duration, 10 volts. Horizontal axis, 1 large division = 5 seconds; vertical axis, 1 large division = 5 mm. of mercury pressure.

rating. In 1 calf, at six months, this suppuration extended to the mediastinum causing the electrode to become nonfunctional. The impression gained so far is that best results are obtained up to about three months.

MANOMETRIC

Spontaneous Motility and Eructation Recordings.—Observed results of recording from the designated positions may be summarized as follows:

a) Reticulum.—Two distinct contractions were regularly recorded, of approximately 8 to 15 mm. of mercury amplitude, having durations of two to three seconds each. A deflection was regularly recorded in conjunction with eructation (graph 1 A).

b) Anterior Dorsal Rumen Sac.—Primary contractions were regularly recorded beginning at about the same time as the second reticular contraction with amplitude of 10 to 20 mm. of mercury, having a duration of approximately five to seven seconds. An eructation deflection also appeared in this tracing (graph 1 A). The secondary contractions, which by palpation involved the anterior pillar as well as more posterior portions of the rumen, were not recorded by a tip placed near the floor of the anterior dorsal sac.

c) Anterior Ventral Rumen Sac.—Primary contractions occurred about ten seconds after the cessation of the second reticular contraction, had an amplitude of approximately 10 to 20 mm. of mercury and a duration of about ten to fifteen seconds. Secondary contractions were not recorded in every cycle. When recorded, these occurred about one to two seconds after pressure increase in the gas pocket in the posterior dorsal sac, having an amplitude of about 2 to 6 mm. of mercury, and a duration of seven to nine seconds. An eructation deflection occurred regularly.

d) Posterior Dorsal Sac (Gas Pocket).—Primary contractions began about the end of the second reticular contraction, had an amplitude of approximately 6 to 12 mm. of mercury and a duration of eight to ten seconds. The secondary contractions, when occurring, were recorded about thirty to sixty seconds following the primary, had an amplitude of 5 to 7 mm. of mercury, and a duration of about five seconds. An eructation deflection appeared regularly.

e) Cardia (1 Inch Anterior to Funnel Opening): Eructations were distinguished from swallowings (1 mark)* by a notched anacrotic limb, were about 10 to 15 mm. of mercury pressure, and had a duration of two to four seconds; swallowings (2 marks)* had, in the great majority of cases, smooth anacrotic and catacrotic limbs, were of about 20 to 40 mm. of mercury amplitude and 1 to 2 seconds duration. Regurgitations resembled swallowings but had broader bases and peaks; all of these data were verified by palpation (intracardial), by direct observation, and by amplification of the esophageal sounds.

Recordings During Dorsal Vagal Trunk Stimulation.—In order to study more adequately specific factors affecting eructation, nitrogen or compressed air was insufflated at constant rates which varied from 5 to 15 liters per minute using a gas flowmeter.**

It was observed in all 3 cows with vagal electrodes implanted that "moderate" stimuli (10 cycles per second frequency, 5 milliseconds pulse duration, 10 volts amplitude applied to the vagus over a two- to five-minute period) were associated with increased contractions of the rumen, particularly from the anterior pillar posteriorly, (graph 1 B) and increased eructation rate; about a twofold increase in eructation rate is seen in graph 1 B.

Easier to obtain was the effect of strong stimuli (graph 2 B). This consisted of spasmodic contractions of the reticulum, rumen, and cardia. Stimuli producing these effects were in general of greater frequency or of greater voltage or both than the "moderate" stimuli noted; for example, an increase to 20 or more cycles per second, or an increase to 30 volts or more would produce the result seen in graph 2 B.

Recordings Before and During Dorsal Vagal Trunk Stimulation, with the Cardia Submerged.—In graph 3 A, the results of infusing 22 to 25 gallons of water into the rumen, and elevating the rear quarters of the animal 10 to 12 degrees in a trailer chute, are seen. The cardia was at this time submerged 6 to 8 inches, as measured by palpation, and about 5 mm. of mercury increase in pressure at the cardia base line occurred. Before recording, the stylus was again placed on the arbitrary base line.

* Signal marker, extreme bottom of tracing.
** Heidbrink Mfg. Co., Minneapolis, Minn.

In 2 of the 3 cows with vagal electrodes (691, 1002), after thus submerging the cardia and closing the fistula opening, eructation was still carried on with or without insufflating extra gas (graph 3 A). These results are in accord with those of Dougherty and Meredith.[14] Further, in these 2 animals, vagus stimulation was associated with a definitely increased eructation rate (graph 3 B).

DISCUSSION AND SUMMARY

1) The intrathoracic electrode technique was applied successfully to 3 adult cows with rumen fistulas.

2) Such animals proved useful for study of motor events and eructation phenomena over a period of three to six months following thoracotomy.

3) Modifications of the techniques described by Brody and Quigley have proved useful for quantitation of motor events in the rumen and reticulum. A special technique for recording eructation at the cardia is described.

4) Stimulation of the dorsal vagal trunk with moderate electrical stimuli was associated, in general, with increase of the rate of contraction in the rumen and increased eructation rate.

5) Stronger electrical stimuli applied to the dorsal vagal trunk were associated, in general, with spasmodic contraction of the rumen, reticulum, and cardia.

6) Elevation of the hindquarters and infusion of large quantities of water served to submerge the cardia 6 to 8 inches. Two of the 3 cows with vagal electrodes were able to eructate regularly under these conditions and increased their eructation rates upon dorsal vagal stimulation.

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Research on Foot-and-Mouth Disease. VIII. Titration of Foot-and-Mouth Disease Virus Making Use of Surviving Tongue Epithelium from Vaccinated Cattle

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VARIOUS investigators[1-3] have devised methods of titration of viruses in tissue culture. By using the tissue-culture method for the growth of foot-and-mouth disease virus in surviving epithelium from tongues of susceptible cattle,[4] Brooksby and Wardle[5] developed a titration method for this virus with the complement-fixation test as an indicator for virus multiplication.

Frenkel[6] showed that the surviving epithelium from tongues of vaccinated cattle supported the growth of foot-and-mouth disease virus in large tissue cultures used for vaccine production and in flask cultures. The present paper describes the use of surviving tongue epithelium from cattle that were vaccinated or immunized with foot-and-mouth disease viruses for the titration of virus by the culture method of Brooksby and Wardle.

STRAINS USED FOR STUDY

Foot-and-mouth disease viruses of types O, A, and C were studied, using strains propagated in tissue cultures, in chicks, and in hens' eggs. The tissue-cultured viruses were Holland type O, Holland type A, and Holland type C, as well as German type C known as the CD strain. Passages from cultures of three sizes were studied. The small cultures were produced in 500-ml. flasks and one half of the tongue epithelium from 1 ox was needed. Tongue epithelium from 40 cattle was required for the medium cultures, and as many as 400 tongues were used in the large cultures. The chick-propagated strains were Holland type A and Holland type C, and the egg strain was Holland type C.

METHODS

The method of Brooksby and Wardle was used to titrate the viruses in surviving tissue.[5] Details are given in their paper. In some tests, modifications or additions were made. For clarity and convenience, a description of the method is included here.

The perspex plates* used were designed by the influenza center of the World Health Organization in England. Each plate has 80 cups. Plastic lids with overhanging ridges fit tightly on the plate and are clamped on by means of rubber bands. A rubber gasket ⅛ in. wide and ⅛ in. thick keeps the lid from the plate surface. The plates are partially sterilized by storage in 10 per cent HCl. Just before use they are washed in tap water, rinsed in distilled water, and dried in a desiccator over H_2SO_4. The plates and lids are exposed to ultraviolet light in a cabinet to complete sterilization. The medium and minced tissue were added in the same cabinet and the lids placed on the plates. Inoculation of the virus dilutions are made using a sterile flat metal box with open ends and a top opening which is of sufficient size to fit over ten exposed cups. The plate is then inserted into a bag made of polyethylene tubing** and sealed with heat.

Buffered glucosol medium[2] containing 1,000 units of penicillin per 1 ml. was routinely used. The buffer was M/25 phosphate solution, pH 7.6, which contained $Na_2H\,PO_4 \cdot 2\,H_2O$ and KH_2PO_4. For the growth of Holland type O tissue-cultured virus, Baker's modified medium[7] also was used. On occasions, 66 μg. per milliliter of streptomycin was added. The medium as well as other fluid test components was placed in the cups with 0.05-ml. dropping pipettes. A spatula was used to introduce the tissue.

The tissue was collected from the tongues of Dutch cattle obtained from the Amsterdam abattoir. These animals had been vaccinated with foot-and-mouth disease tissue-culture vaccines representing types O, A, and C within two to twenty-six weeks before slaughter. The tongues were fresh and warm upon arrival at the laboratory and the epithelium was harvested by one of two methods, both developed by Frenkel. When the clamp method[7] was applied, only the underlying mucosa that was exposed was sliced from 2 or 3 tongues with a safety-razor blade in a holder. The second method employed a Berkel slicing machine.[8] The topmost horny layer of the mucosa was sliced and discarded, then the slicing was continued until the bluish submucosal tissue was visible. These

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* The plates are obtainable from Messrs. Prestware Ltd., Lombard Rd., London SW 19, England.

** Supplied by BK Plastics Ltd., Chingford, London, England.

epithelial sheets were retained. By this method, only a portion of two to three tongues was used for mincing. With each method care was exercised to exclude underlying muscle tissue, which becomes acid and has harmful effects on virus growth. The epithelium was placed in 100-ml. centrifuge tubes and buffered glucosol or modified Baker's medium added. The tissue was minced with scissors to make 1-mm. cubes. On a weight per volume basis the tissue was washed, unless otherwise stated, seven or eight times with a volume of buffered medium 100 to 125 times its weight. From 12 to 16 mg. of washed tissue were placed in each cup.

The virus dilutions were prepared in the medium used in the test. The dilutions ranged from 10^{-3} to 10^{-7}; 0.1 ml. of each dilution was inoculated into 10, 20, 30, 40, or 50 cups.

The plates were clamped on a shaking machine that was used primarily for flask tissue cultures and kept in an incubation room at 37 C. This type of shaking action gave satisfactory results. Incubation and agitation were continued for sixty-five hours at 37 C.

The complement-fixation test was employed to test for virus. A suitable dilution of complement sufficient to give 100 per cent hemolysis in the presence of specific antiserum and tissue was added. Usually 0.1 ml. of a 1:20 or 1:30 dilution of complement was used with each test group and this represented 1½ to 3 complete hemolytic units of complement. Then 0.1 ml. of a 1:8 dilution of the appropriate guinea pig hyperimmune serum was added. The plate was placed in a water bath at 37 C. for thirty minutes. A 1.7 per cent suspension of maximally sensitized cells was prepared and placed in a water bath for fifteen minutes at 37 C. just before the addition of 0.1 ml. to each cup. Incubation of the plates was continued for forty-five minutes with the tissue-cultured strains and early-passaged chick strains. The reading of the test can be made about ten minutes after removal from the water bath or after holding at 4 C. for four or more hours (fig. 1). Any degree of fixation is a positive reaction. With the thirtieth transfer of Holland type A chick-propagated virus and the egg-propagated strain, it was necessary to make a reading after five to ten minutes of incubation in the water bath. This made the test less reliable since it was more difficult to read.

Fifty per cent end points in tissue culture ($t.c.i.d._{50}$) and in cattle ($i.d._{50}$) were calculated by the method of Reed and Muench.[9]

The method of Henderson[10] was employed to titrate the virus in cattle. In this case, virus dilutions were prepared with a M/25 buffered phosphate solution, pH 7.6. With the chick and egg strains the dilutions ranged from 10^{-4} to 10^{-7}. Two animals were used, each dilution was inoculated into ten tongue sites, five per ox.† With the tissue-cultured strains, the test dilutions were 10^{-5} and 10^{-6} and each dilution was inoculated into ten or 20 sites using 1 or 2 animals.

† The oxen used in titration studies were imported from Ireland.

TABLE 1—Comparative Titrations of Foot-and-Mouth Disease Viruses in Cattle and in Tissue Culture with Surviving Tongue Epithelium from Vaccinated Cattle

Virus type	Origin	Passage No.	Culture titration		Cattle titration	
			No. of sites/dil.	$i.d._{50}$-log	No. of sites/dil.	$i.d._{50}$-log
C	L.C.	84	20	4.9	20	5.0
C	L.C.	99	30	6.0	20	5.5
C	S.C.	87	20	5.3	10	6.0
C	S.C.	90	20	5.6	10	5.0
C	S.C.	91	20	5.1	10	5.7
C	S.C.	92	20	5.4	10	5.3
C	S.C.	93A	40	6.5	10	5.8
C	S.C.	93B	10	5.8	10	5.1
C	S.C.	96	30	5.8	10	5.2
CD*	S.C.	2	20	5.6	10	5.8
CD	S.C.	3	30	5.6	20	5.6
CD	S.C.	5	10	5.3	20	5.4
CD	S.C.	6	30	5.0	20	5.8
CD	S.C.	7	20	5.6	20	5.5
CD	S.C.	8	20	5.8	20	5.6
A	L.C.	280	30	5.2	20	5.7
A	L.C.	326	50	5.3	20	5.6
A	S.C.	309	20	5.6	10	6.0
A	S.C.	311	20	5.4	10	5.4
A	M.C.	309	20	5.6	10	5.8
A	M.C.	310	20	5.9	10	5.4
A	M.C.	311	20	6.2	10	6.2
A	M.C.	312	10	5.4	20	6.0
A	M.C.	314	20	5.8	20	5.6
A	M.C.	316	20	6.6	20	5.9
C	Egg	11	10	5.8	10	5.3
A	Chick	10	10	5.6	10	5.9
A	Chick	30	10	6.0	10	6.0
C	Chick	5	10	6.8	10	5.6

L.C.—large culture; M.C.—medium culture; S.C.—small culture. * German type C virus.

The tissue-cultured virus suspensions that were filtered through an EK Seitz filter were kept at 6 C. and usually titrated in cattle within one to two days after the culture was harvested. The titrations in tissue culture were performed within six days of the cattle test. The heart muscle pool of 4 chicken embryos and gizzard muscle pools of 3 to 5 chicks were kept at —15 C. until titrated in

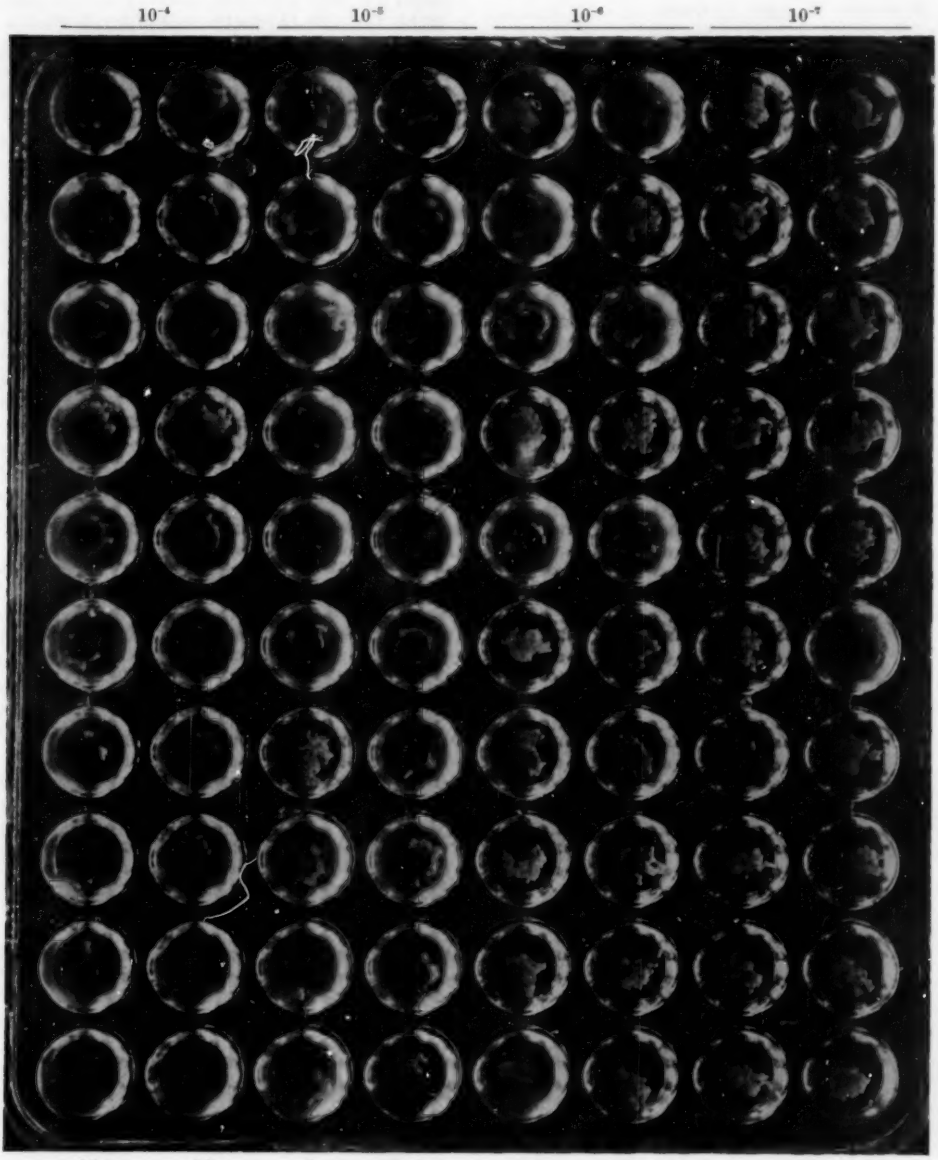

Fig. 1—Dilutions of foot-and-mouth disease virus. In the positive cups, the red cells can be seen as a definite button which obscures the tissue fragments. In the negative cups, the tissue fragments are clearly seen and red cells are lysed.

cattle and in tissue culture. The tissues were thawed and made into a 10 per cent dilution in buffered glucosol by a mortar, pestle, and sterile sand. After centrifuging at 3,500 r.p.m. for ten minutes, the supernatant was decanted and used to prepare the virus dilutions.

RESULTS

Comparative Titrations in Cattle and in Tissue Culture.—The epithelial tissue was collected by the clamp method from the tongues of Dutch cattle that had been vaccinated with types A, O, and C foot-and-mouth disease tissue-culture vaccines. Small, medium, and large cultures of Holland type A tissue-cultured virus and small and large cultures of Holland type C tissue-cultured virus were tested, as well as small cultures of the German type C tissue-cultured strain. Holland type A and Holland type C chick-propagated foot-and-mouth disease viruses and Holland type C egg-propagated foot-and-mouth disease virus also were titrated in cattle and in tissue culture (table 1).

In tests with 10 different passages of Holland type A tissue-cultured virus, the virus had end points ranging from $10^{-5.2}$ to $10^{-6.6}$ in tissue culture and from $10^{-5.4}$ to $10^{-6.2}$ in cattle. By the two titration methods, the greatest difference in end points with a single transfer was 0.7 log unit. In tests with nine transfers of Holland type C tissue-cultured virus, end points ranged from $10^{-4.9}$ to $10^{-6.5}$ in tissue culture and from $10^{-5.0}$ to $10^{-6.0}$ in cattle, with the greatest comparative difference 0.7 log unit. In tests with 6 transfers of German type C tissue-cultured virus (CD strain), the virus had end points ranging from $10^{-5.0}$ to $10^{-5.8}$ in tissue culture and from $10^{-5.4}$ to $10^{-5.8}$ in cattle; the greatest comparative difference was 0.8 log unit.

Gizzard muscle infected with type A virus when titrated in cattle had an end point of $10^{-5.9}$ for the tenth transfer and $10^{-6.0}$ for the thirtieth transfer in chicks. By tissue culture, titration end points were $10^{-5.6}$ for the tenth transfer and $10^{-6.0}$ for the thirtieth transfer. Gizzard muscle from the fifth transfer of type C virus had an end point of $10^{-5.6}$ in cattle and $10^{-6.8}$ in tissue culture.

Heart muscle of 4 chicken embryos infected with type C virus and representing the eleventh transfer in hens' eggs had an end point of $10^{-5.8}$ in tissue culture and $10^{-5.3}$ in cattle.

Comparison of the Influence of Different Methods of Collecting the Tissues on the Outcome of the Test.—Tongue epithelium from vaccinated Dutch cattle was collected by the clamp method and by the Berkel machine method. A number of culture passages of Holland A and C and German C viruses were titrated in duplicate, using tissues collected with each of the methods mentioned. The same cultures also were titrated in cattle (table 2).

In titrations of 14 different transfers with tissue collected by the clamp method, the tissue cultured viruses had end points ranging from $10^{-4.9}$ to $10^{-6.5}$. When titrated in cattle, the transfers had end points ranging from $10^{-5.1}$ to $10^{-6.2}$. By the two procedures, the greatest difference in virus end points with a single transfer was 0.9 log unit.

Comparison of Titrations of Holland Type O Tissue-Cultured Virus in Tissue Culture with Buffered Glucosol Medium and with Modified Baker's Medium, and in

TABLE 2—Comparison of the Clamp Method and the Berkel Method of Collecting Surviving Tongue Epithelium from Vaccinated Cattle in Tissue-Culture Titration of Virus

Type virus	Culture size	Passage No.	Culture titrations $i.d._{50}$ log		Cattle titration $i.d._{50}$ log
			Clamp method	Berkel method	
A	Small	311	5.4	5.8	5.4
A	Medium	309	5.6	5.5	5.8
A	Medium	310	5.5	5.9	5.4
A	Medium	311	6.2	5.3	6.2
A	Medium	313		5.9	5.8
A	Medium	314	5.8	4.9	5.6
C	Small	87	5.3	6.0	6.0
C	Small	90	5.6	6.5	5.0
C	Small	91	5.1	6.0	5.7
C	Small	92	5.4	6.0	5.3
C	Small	93	6.5	6.3	5.8
C	Small	94	5.8	5.5	5.1
CD	Small	2	5.6	4.9	5.8
CD	Small	3	5.6	5.6	5.6

Cattle.—The 244th, 253rd, 296th, 298th, and 302nd transfers of Holland type O tissue-cultured virus were titrated in cattle and in tissue culture using glucosol medium. The 244th and 302nd transfers also were titrated in tissue culture using modified Baker's medium (table 3).

In tissue culture using glucosol medium, the five transfers had end points ranging from $10^{-3.4}$ to $10^{-4.5}$. The same transfers in cattle had end points ranging from $10^{-5.7}$ to $10^{-6.1}$. By these two methods, the minimum difference between end points of a single transfer was 1.5 log units, the maximum difference was 2.7 log units. With modified Baker's medium in tissue culture, the 244th transfer had an end point of $10^{-6.0}$ and the 302nd transfer had an end point of $10^{-5.5}$. Tissue-culture titration results with modified Baker's medium correlated well with cattle titrations of these two transfers.

Use of Surviving Epithelium in Tissue Culture from the Tongue of an Ox Recovered from Foot-and-Mouth Disease.—Epithelium from the tongue of an animal that had been used in a titration of Holland type A culture virus in cattle was collected by the clamp method 6 weeks after inoculation. The tissue was divided into equal portions, 1 portion being washed with cold buffered glucosol with a volume ten times its weight and the other with a volume 100 times its weight. Then the tissues and buffered glucosol were added to the cups and virus dilutions of the 323rd transfer of Holland type A tissue-cultured virus were added. Ten sites were used per dilution for each kind of washed tissue. This same passage was titrated in 2 susceptible oxen (table 4).

In the tissue-culture test with the tissue washed with a volume ten times its weight, the virus had a poor titration distribution and an end point of $10^{-3.9}$. In contrast, the virus in tissue culture with the same tissue washed with a volume 100 times its weight had an excellent distribution and an end point of $10^{-6.0}$. The titration in cattle yielded an i.d.$_{50}$ of $10^{-5.6}$.

TABLE 3—Comparison of Titrations with Holland Type O Tissue-Cultured Virus in Tissue Culture with Buffered Glucosal Medium and with Modified Baker's Medium and in Cattle

Passage No.	Tissue mediums	Titration i.d.$_{50}$-log	
		Tissue culture	Cattle
244	Glucosal	3.4	6.1
	Modified Baker's	6.0	
253	Glucosal	3.6	5.9
296	Glucosal	3.6	5.7
298	Glucosal	4.4	6.1
302	Glucosal	4.5	6.0
	Modified Baker's	5.5	6.0

The Use of Tissue Culture for the Titration of Serum-Neutralizing Antibodies in Serums of Chickens and Cattle.—The methods and results of the serum neutralization tests in tissue culture with serums of chickens and cattle are given in previous papers.[11,12] The convalescent serums of cattle were taken seventeen days after inoculation with foot-and-mouth disease virus and those from chickens were taken twenty-eight days after inoculation. Briefly, the results were as follows: In tests with chicken serums, virus without serum had a t.c.i.d.$_{50}$ of $10^{-5.1}$. The preinoculation serums of 3 chickens that were inoculated with type A chick virus were diluted 1:10; the virus-serum mixtures had end points ranging from $10^{-5.3}$ to $10^{-5.5}$. The convalescent serums of 4 chickens were diluted 1:10 and the virus-serum mixtures had end points ranging from $10^{-2.7}$ to $10^{-4.9}$. In the first test with serums taken from 2 cattle that were inoculated with type C egg virus, the virus without serum had a t.c.i.d.$_{50}$ of $10^{-5.8}$ and identical end points of $10^{-5.9}$ were obtained with the two preinoculation serums that were diluted 1:10. With the two convalescent serums that were diluted 1:10, end points of less than t.c.i.d.$_{50}$ $10^{-0.5}$ were read. In a repeat test, the virus without serum had an end point of $10^{-5.7}$ whereas with preinoculation serums diluted 1:10, the end points

TABLE 4—The Effects of Washing Surviving Epithelium from the Tongue of an Ox Recovered from Foot-and-Mouth Disease on the Titration of Virus in Tissue Culture

Washing dilution*	Tissue culture						Cattle	
	Dilution of virus							
	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	i.d.$_{50}$-log	No.	i.d.$_{50}$-log
1:10	8/10**	2/10	5/10	0/10	1/10	3.9	1	5.6
1:100	10/10	10/10	10/10	5/10	0/10	6.0	2	5.6

* Based on weight-volume relationship; ** numerator indicates the number of positive cultures, denominator indicates the number of cultures tested.

were $10^{-6.3}$ and $10^{-6.4}$. With the convalescent serums diluted 1:10 and 1:100, end points of less than t.c.i.d.$_{50}$ $10^{-0.5}$ were noted.

DISCUSSION

Brooksby and Wardle successfully applied the tissue-culture method in the titration of foot-and-mouth disease virus of cattle origin or tissue-culture origin, and in comparative titrations in culture and in cattle good agreement was obtained with four of the five strains tested.[5] With the fifth strain, the difference between the two methods exceeded 0.9 log unit estimated by Henderson as the significant difference between the results of two titrations each based on ten observations for each of four tenfold dilutions in 2 cattle.[10] In our studies, we confirmed the reliability of the tissue-culture method of Brooksby and Wardle and obtained good agreement in comparative titrations in tissue culture and in cattle with three tissue-culture strains, two chick-propagated strains and one egg-propagated strain. By the use of modified Baker's medium instead of glucosol, good agreement also was observed with Holland type O tissue-cultured foot-and-mouth disease virus. Furthermore, it was shown that surviving epithelium from tongues of vaccinated and immune cattle could be successfully used in these titration studies of the virus. These findings were anticipated since foot-and-mouth disease virus grew well in larger tissue cultures with surviving tongue epithelium from vaccinated cattle. Experimental results showed, however, that the tissue from immune cattle must be properly washed to give satisfactory results. The epithelial tissues collected from the tongues of vaccinated cattle by the clamp method gave slightly higher end points in the titrations than tissue harvested with the Berkel slicing machine.

With early passages of Holland types A, O, and C chick-propagated foot-and-mouth disease viruses, good results were observed in tissue-culture titrations of these strains. With later passages of the chick viruses and type C egg virus, less reliable results were obtained. Tissue-cultured viruses titrated at the same time gave good results. Consequently, poor selection of complement dose or poor guinea pig antiserum can not account for inferior results with later passages of the chick-propagated strains. These data have been presented in this investigation and in earlier studies.[11-13].

SUMMARY

Surviving epithelium from tongues of cattle that were vaccinated against foot-and-mouth disease and from animals that had recovered from the infection was used successfully in the tissue-culture titration method of Brooksby and Wardle. In comparative titrations in cattle and in tissue culture with four tissue-culture strains, two chick-propagated strains, and one egg-propagated strain, good correlation was observed. With one tissue-culture strain, modified Baker's solution instead of glucosol was used to obtain satisfactory results. Epithelium collected by the clamp method or Berkel machine method can be used in tissue culture.

Virus neutralizing antibodies produced in chickens and in cattle after infection with foot-and-mouth disease could be detected using the tissue-culture titration method.

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Preliminary Studies on Pseudomonas Mastitis

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BACTERIA of the genus Pseudomonas are widely distributed in surface water and fertile soil. Early workers[1] considered Pseudomonas a saprophyte associated with decomposition of organic material and as a secondary invader in wound infections. In recent years, it has been considered a pathogen associated with many diseases of man and animals. The earliest report incriminating Pseudomonas as a cause of mastitis was by Mundhenk.[2] Currently, Pseudomonas mastitis is being recognized in an increasing number of herds. It presents a serious problem because no consistently successful method of control or treatment has been developed.

In an affected herd, cases occur sporadically and intermittently and the bacteria count of the market milk is high. In an affected quarter the secretion may vary from a straw-colored, watery fluid to a thick, purulent material. The duration of an attack is usually not more than two or three days, and the quarter may return to normal with no treatment other than thorough milking. However, in both treated and untreated quarters which have apparently returned to normal, the infection may become active again.

MATERIALS AND METHODS

Culture Studies.—A total of 2,629 quarter-milk samples were cultured and examined for type of bacterial growth using the following procedure.

Collection.—Samples were collected just before the regular milking time. The udder was washed and dried and the teat orifice cleaned with cotton and 70 per cent alcohol, left to right side. Milk was collected from the right to the left side. The first few milliliters were discarded, then 10 ml. of fore milk was collected in a sterile 15-ml. screw-cap tube.

Laboratory Procedure.—Samples were incubated twelve to fourteen hours at 37 C. Incubation was started not more than twelve hours after collection. After incubation, milk was streaked on 10 per cent bovine blood agar plates which were incubated at 37 C. for twenty-four hours, then twelve to sixteen hours at 6 to 10 C.

Serological Studies.—Antigen was prepared from a culture of *Pseudomonas aeruginosa* isolated from an animal with an acute case of mastitis.

A 24-hour culture was removed from a Kolle flask with 10 ml. of sterile 0.85 per cent sodium chloride containing 2 per cent formalin and cultured for sterility. Stock antigen was diluted with 0.85 per cent NaCl to contain ten times tube No. 3 of McFarland's nephelometer (9 billion organisms per cubic centimeter). Antigen for the test proper was diluted 1:10 with 0.85 per cent NaCl.

Milk serum was removed by coagulation with rennet* within twelve hours after collection.

Serum titers were determined by the presence or absence of macroscopic agglutination after incubation at 37 C. for forty-eight hours. Twofold dilutions from 1:10 to 1:2560 were used. All tests were run in duplicate.

Blood and quarter-milk samples were collected from 32 control animals with no clinical signs of mastitis from herds where Pseudomonis organisms were never isolated; from 96 animals known to be infected with Pseudomonas; from 21 animals with no clinical signs of mastitis but in herds infected with Pseudomonas; and from 24 animals which showed clinical signs of mastitis from which micrococci, coliform organisms, or Proteus organisms were cultured.

Antibiotic Sensitivity Studies.—For comparative studies, five mediums were used: (1) for the control, brain heart infusion broth (Difco); (2) pooled, unheated Pseudomonas mastitis milk from 4 animals with clinical mastitis which previously had been positive on culture for *Ps. aeruginosa;* (3) pooled Pseudomonas milk heated for sterilization; (4) sterile, fat-free milk; (5) normal, unheated milk.

The antibiotics used were neomycin, streptomycin, chloramphenicol, polymyxin B, chlortetracycline, and oxytetracycline.

The organism used was *Ps. aeruginosa* isolated from 4 animals with acute cases of mastitis and pooled after culturing. The concentration of organisms in all test mediums was 10,000 organisms per milliliter.

The test tube method, with twofold dilution, was used in all the tests. All tests were run in duplicate. Each antibiotic was diluted twofold from 400.0 μg. per milliliter to 0.002 μg. per ml. Evidence of growth was observed by pigment production and checked by subcultures. All tests were incubated at 37 C. and checked at twenty-four,

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* The rennet used was "junket"; 1 tablet was dissolved in 100 ml. of distilled water. One milliliter of this solution was added to 5 ml. of milk and allowed to stand at room temperature for twelve hours.

forty-eight, and seventy-two hours. The variation between the two duplicate samples in all the series was not greater than one dilution.

RESULTS

In our laboratory, organisms showing the following characteristics are classified as Pseudomonas:

a) gram-negative motile rods;
b) an odor of trimethylamine;
c) acid from glucose, not from lactose;
d) urease negative;
e) presence or absence of green pigment;
f) colonial characteristics—one of the following:
1) Spreading—lobulated edges, smooth, convex, dark centers, translucent edges;
2) nonspreading—smooth, convex, dark centers, translucent edges;
3) rough—round or slightly irregular edges, finely or coarsely granular.

Table 1 shows the cultural characteristics of the 147 isolations of Pseudomonas.

The majority of the isolated organisms produced spreading colonies. All had a detectable grapelike odor of trimethylamine, although this odor was less pronounced in the 29 nonpigmented organisms.

Pseudomonas organisms were found in the water supply to the barn and to the milk house in three herds infected with Pseudomonas.

We have found that in Pseudomonas mastitis, the causative agent can not be isolated each time a milk sample is cultured. Of 164 samples collected from two known positive quarters, Pseudomonas organisms were isolated only 42 per cent of the time (table 2).

The 32 control animals and the 24 animals with other types of mastitis showed no titer above 1:40 in either blood or milk serum.

TABLE 1—Characteristics of Pseudomonas

No. of isolates	Large spreading colony	Round colony	Rough colony	Odor	Gram-negative motile rod	Glucose (acid)	Lactose	Urease	Pigment
118	65	37	16	118	118	118	0	0	118
29	12	11	6	29 slight	29	29	0	0	0

In the 96 animals with at least one quarter shedding Pseudomonas, the blood serum titers ranged from 1:160 to 1:1280. In these 96 animals, 147 quarters showed positive cultures with an elevated milk titer (ranging from 1:80 to 1:640). The 237 quarters which were negative for Pseudomonas on at least two culturings had milk serum titers ranging from 1:20 to 1:320. Further work is necessary to interpret the significance of milk serum titers.

The 21 animals from infected herds, from which Pseudomonas was not isolated on two repeated culturings, showed blood serum titers from 1:80 to 1:320 and milk serum titers from 1:20 to 1:320. In herds infected with Pseudomonas, 18 animals did not show elevated blood or milk serum titers.

A blood serum titer of 1:160 or higher was noted in all animals shedding Pseudomonas. The milk serum titer in all quarters shedding Pseudomonas was 1:80 or higher. It took up to four repeated culturings to isolate Pseudomonas from some of 147 quarters of the animals with blood serum titers of 1:160 or higher. On the first examination of the group of 96 animals, all had blood serum titers of 1:160 or

TABLE 2—Blood and Milk Serum Titers for Pseudomonas Aeruginosa

Type of animals	No. of animals	Blood serum titer	Quarters yielding any Pseudomonas		Quarters negative on culture to Pseudomonas (cultured twice)	
			No. quarters	Milk titer	No. quarters	Milk titer
Pseudomonas mastitis	96	1:160–1:1280	147*	1:80–1:640	237	1:20–1:320
Animals in infected herd	21	1:80 –1:320	0	.....	84	1:20–1:320
Normal	32	1:20	0	.....	128	1:20
			Other organisms			
Other types mastitis	24	1:20	71	1:40	25	1:40

* Organisms shown in table 1.

TABLE 3—In Vitro Antibiotic Concentrations Required to Prevent Growth of Pseudomonas Aeruginosa in Different Testing Mediums

Type of medium	Neomycin*		Streptomycin*		Polymyxin B*		Chloramphenicol*		Chlortetracycline*		Oxytetracycline*	
	24 hr.	72 hr.	24 hr.	72 hr.	24 hr.	72 hr.	24 hr.	72 hr.	24 hr.	72 hr.	24 hr.	72 hr.
Broth	12.5	25.0	3.12	12.5	0.04	0.19	0.39	1.56	6.25	12.5	6.25	25.0
Mastitis milk (unheated)	50.0	200.0	50.0	100.0	3.12	25.0	6.25	25.0	50.0	100.0	200.0	400.0
Sterile milk (unheated)	25.0	100.0	6.25	50.0	0.78	6.25	3.12	12.5	12.5	25.0	50.0	100.0
Mastitis milk (heated)	25.0	200.0	12.5	50.0	0.09	0.78	1.56	12.5	25.0	100.0	50.0	200.0
Nonfat milk (heated)	25.0	50.0	6.25	25.0	0.09	0.39	0.78	3.12	6.25	25.0	6.25	50.0

* Concentration microgram per milliliter; range from 24 to 72 hours.

higher, but only 86 quarters of 62 animals yielded Pseudomonas on culture.

Table 3 shows the concentration of antibiotics which prevented growth at twenty-four and seventy-two hours.

There is an increase in the amount of each antibiotic needed for the inhibition of growth in milk mediums as compared with broth controls.

The unheated mastitis milk required the highest concentration of all the antibiotics to inhibit growth.

The concentration required to inhibit growth in broth and milk mediums was nearly the same in the case of neomycin but concentrations needed to inhibit growth in milk mediums was the lowest with polymyxin B and chloramphenicol.

Polymyxin B gave similar results in broth and heated milk samples, while in the unheated milk it was considerably less effective.

DISCUSSION

The identification of bacteria of the Pseudomonas group can be confusing because of conflicting opinions. Many investigators consider pigment production an essential characteristic and classify only the strains which produce chloroform-soluble pigment as *Ps. aeruginosa*.[3,4]

We have found that many strains either do not have or have lost their ability to produce pigment. This observation has been reported by other workers.[7-9]

Blood and milk serum agglutination titers for Pseudomonas have been shown to be aids in the diagnosis of Pseudomonas infection.

Milk which has been incubated for bacteriological examination can not be used for separation of serum, as false negatives will be obtained. If double milk samples are not collected, it is necessary to divide the samples, and to use part for serological study and the other part for bacteriological work.

Blood serum should be removed from the clot without delay as hemolyzed blood gives false positive reactions.

In vitro antibiotic sensitivity results are helpful in the treatment of many infections, but not all *in vitro* results are reproducible *in vivo*. One of the many reasons for this is the fact that the blood agar or broth used in sensitivity testing is unrelated to living tissue. In mastitis, milk is the substance in which the bacteria are growing and into which the antibiotic is injected. It is possible that some constituent of the milk may interfere with the action of the antibiotic. This may explain why an antibiotic highly effective *in vitro* does not produce the expected clinical results in the treatment of mastitis.

SUMMARY

1) Organisms may be classified as Pseudomonas even though no pigment is detected.

2) Random culturing of milk will not show all quarters harboring Pseudomonas organisms.

3) Blood agglutination titers may be correlated with cultural results.

4) Milk agglutination titers are of possible value in diagnosing Pseudomonas infection.

5) Mastitis milk is the medium of choice in antibiotic sensitivity testing.

CONCLUSION

Pseudomonas mastitis is a problem in an increasing number of herds. Since Pseudomonas organisms are not shed regularly

in the milk, blood and milk serum titers may aid in diagnosing its presence in affected herds.

Further investigation, particularly of the water supply, is needed regarding the source of infection.

On the basis of *in vitro* tests using milk medium, chloramphenicol, polymyxin B, and neomycin appear to be the antibiotics of choice for clinical trials in the treatment of Pseudomonas mastitis.

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Statement of Ownership and Management Required by the Acts of Congress of August 24, 1912, as amended by the Acts of March 3, 1933, and July 2, 1946, for the American Journal of Veterinary Research. Published Quarterly at Chicago, Ill., as of Oct. 1, 1948: "State of Illinois, County of Cook, before me, a notary public, in and for the state and county aforesaid, personally appeared Dr. J. G. Hardenbergh, who having been duly sworn according to law, deposes and says that he is the managing editor of the American Journal of Veterinary Research and that the following is to the best of his knowledge and belief a true statement of the ownership and management of the aforesaid publication for the date shown in the above caption, required by the Act of August 24, 1912, as amended by the Act of March 3, 1933, embodied in section 537, Postal Laws and Regulations, to wit:

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"Sworn to and subscribed before me this Fifteenth day of September, 1955:
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Resistance of Calf Scour-Producing Organisms to Broad Spectrum Antibiotics

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THE EVER increasing reports of different bacterial strains, which are developing resistance to the broad spectrum antibiotics, have been the subject of numerous reports. Rodman [1] reports that long-term use of these agents may cause a "superinfection" of a strain which has developed resistance to the action of the antibiotics. Weinstein *et al.*[2] report that a majority of the resistant organisms in the intestinal tract are strains which have developed resistance to the antibiotics during oral use.

Since the action of the broad spectrum antibiotics is bacteriostatic, it was decided to try a combination of bactericidal antibiotics which would not permit the development of resistance and which would have a large range of activity.

The purpose of this paper is to present some data obtained in our attempt to find an effective bactericidal combination of antibiotics, which would prevent the development of bacterial resistance to its action.

METHODS AND MATERIALS

The organisms tested here were isolated from animals with active cases of calf scours. The disease had developed after the animals had been treated for other diseases with broad spectrum antibiotics. The organisms isolated were *Pseudomonas aeruginosa*, *Escherichia coli*, and a Salmonella species.

The antibiotics tested were oxytetracycline, tetracycline, chlortetracycline, neomycin, and polymyxin B, alone and in combination.

The medium used for culturing the organisms contained yeast extract, 2.0 per cent; peptone, 0.5 per cent; dextrose, 1.0 per cent; monosodium phosphate, 0.2 per cent; and sorbitan monooleate complex, 0.1 per cent, at a pH of 6.8. The test medium contained beef extract, 0.5 per cent; peptone, 1.0 per cent; and sodium chloride, 0.25 per cent, at a pH of 6.9 to 7.0.

The test medium was made in double strength concentrations and placed in 5-ml. amounts in metal-capped test tubes (18 x 150 mm.). The tubed medium was then sterilized at 121 C. for fifteen minutes, cooled, and aseptically diluted to 10 ml. with the antibiotic solution. The tubes were then inoculated with 0.1 ml. of a twelve-hour culture of the test organism, and were incubated for twenty-four hours at 37 C. At the end of this period, the tubes were checked for growth.

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RESULTS

Each organism tested displayed a strong resistance to the tetracycline group of broad spectrum antibiotics (table 1). These same organisms were inhibited by much lower concentrations of neomycin or polymyxin B alone and by an even lower concentration when these two antibiotics were combined.

Neomycin and polymyxin B displayed a synergistic action when in combination.

DISCUSSION

There appears to be a cross-resistance pattern displayed by bacteria to the tetracycline groups of antibiotics.

A synergistic action appears to be exerted on these organisms when neomycin and polymyxin B are combined. This is probably brought about by the different modes of action of the two bactericidal agents.[3]

The uncontrolled use of broad spectrum antibiotics in the field of veterinary medi-

TABLE 1—Concentration (μg./ml.) of Antibiotics Required to Inhibit the Organisms

Organisms	Individual Antibiotics					Combination	
	Oxytetracycline	Tetracycline	Chlortetracycline	Neomycin	Polymyxin*	Neomycin	Polymyxin*
Pseudomonas aeruginosa	1,000	1,000	1,000	12.0	2.5	1.0	1.0
Escherichia coli No. 1	1,000	1,000	500	36.0	2.1	0.6	0.6
Escherichia coli No. 2	1,000	1,000	1,000	28.5	3.0	1.5	1.5
Escherichia coli No. 3	350	500	1,000	36.5	2.5	1.25	1.25
Salmonella spp.	250	250	500	22.5	3.8	1.4	1.4

* Each milligram is equal to 10,000 units of polymyxin B.

cine appears to be creating a group of organisms which are becoming resistant to these agents. The action of these broad spectrum antibiotics is primarly bacteriostatic and the surviving organism is thus allowed to develop a resistance to these antibacterial agents rather rapidly.

Neomycin and polymyxin B both possess a bactericidal action, and susceptible bacteria, such as those causing calf scours, do not develop a resistance to the action of these agents.[4, 5, 6]

SUMMARY AND CONCLUSIONS

Five organisms isolated from calf scours were tested for susceptibility to five antibiotics. A cross-resistance pattern to the tetracycline group was shown to exist. A synergistic action was shown to exist between neomycin and polymyxin B.

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Neutralization of Bovine Papilloma Virus with Serums from Cattle and Horses with Experimental Papillomas

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THE RESISTANCE of cattle to reinfection with cutaneous papilloma has been known for a long time,[1-3] but the mechanism of this resistance is not thoroughly understood. This report deals with two experiments testing the neutralizing ability of various serums obtained during the course of studies with papilloma infections in cattle and in horses.

MATERIALS AND METHODS

Papilloma Viruses.—Strains 30 and 200 of bovine papilloma were used in the experiments. Strain 30 originated from a continuous growth excised from a steer in 1951. Strain 200 was obtained in 1953 from a Shorthorn heifer at the time of slaughter. The tissues were stored at 5 C. in 50 per cent glycerin. On the day the serum neutralization tests were performed, a portion of the growth was weighed, ground in a mortar, and suspended in 10 parts physiological salt solution. The suspension was centrifuged at low speed for five minutes and the sediment discarded. A small amount of crystalline penicillin G was added to the supernatant fluid. A clean pipette was used for making each dilution of the inoculum to avoid transfer of excess virus. The virus preparations were titrated for infectivity in the horses at the same time the neutralization tests were made. The dilutions for titration ranged from 1:10 to 1:100,000,000.

Test Serums.—Serums from 20 calves were used in one serum neutralization experiment. Sixteen of the 20 calves had been injected with wart vaccines before the pre-exposure serum samples were obtained. After the first blood samples were taken, the 20 calves were exposed to papilloma agents (strains 30 and 200). One month after exposure, the calves were bled again. At this time, 13 calves had warts at one or more inoculation sites. Two months after exposure all but 1 animal had warts. The serums were stored at —60 C. until used.

Serums which were obtained from 2 horses with papillomas and 2 with no previously known papillomas were used for another neutralization experiment. Two of the horses (32 and 33) each had fifty growths experimentally produced by strain 30. Immunity of the horses was challenged by inoculation with homologous material at different times. Horse 32 was immune fifteen days after the original exposure and also resisted a challenge inoculation four months later. Horse 33 developed slight growths following challenges made at five-day intervals from ten to thirty days after the first exposure but was immune four months later. The serums used in the neutralization test were obtained from the horses three months after the original exposure. Nonimmune serums were obtained from horses 34 and 35 (later used as test horses) which had no history of previous papilloma infection. A portion of each serum was heated to 56 C. for thirty minutes and stored at —60 C. until used.

Test Animals.—Susceptible horses were used as test animals because they have a more prompt and consistent reaction to the bovine papilloma agent than cattle.[4] The body surface of a horse was also more convenient for a large number of inoculation sites (fig. 1). Horses 37 and 38 were used to test the neutralizing capacity of cattle serums and horses 34 and 35 were the test animals for horse serums.

Neutralization Tests.—Cattle serums were tested against both strains 30 and 200, diluted 1:100. The serums from the first bleeding were diluted 1:10, 1:40, 1:80, and 1:160, and serums

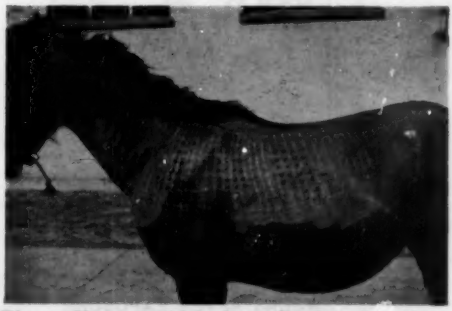

Fig. 1—Horse 37, thirty days after intradermal exposure to mixtures of cattle serums and bovine papilloma virus. The 188 skin-test areas are outlined with lacquer. Duplicate inoculations were made on the other side of the horse. Lack of neutralization resulted in growths. Larger growths from the more active strain 200 may be noted in half of the skin-test areas. In the remainder of the skin-test areas, strain 30 had produced only slight reaction at the time of this photograph but subsequently became more prominent.

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from the second bleeding were diluted 1:10, 1:40, 1:320, and 1:1280. The wide difference in dilutions was to avoid negative prozone reactions. The mixtures of equal volumes of virus and serum were allowed to stand for one hour at room temperature. Duplicate intradermal injections were made on horses 37 and 38 and the sites of inoculation were examined weekly for six weeks.

Horse serums were tested with two dilutions of agent 30 (1:100 and 1:10,000). Each dilution of the agent was tested against both heated and unheated serum diluted to 1:4 and 1:16 in saline. Mixtures of equal volumes of virus and serum were allowed to stand for one hour at room temperature. Duplicate intradermal injections of approximately 0.01 ml. were made on horses 34 and 35. The inoculation sites were examined once a week for four months and the diameter of the growths recorded.

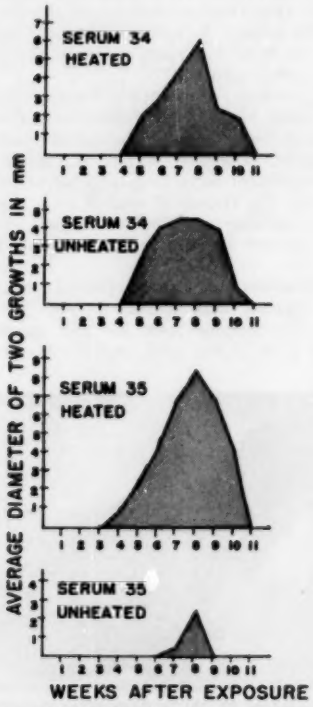

Fig. 2—Graphic illustration of the diameter and duration of growths obtained on horse 35 following intradermal inoculation of bovine papilloma agent (strain 30), diluted 1:100 and mixed with heated and unheated normal horse serum diluted 1:4. Note the inhibition of growth by unheated serum from horse 35, which was not shown by heated serum. The serum was obtained prior to experimental inoculation. This effect was not obtained with serum from horse 34.

RESULTS

Growths developed from intradermal inoculation of mixtures of cattle serums and papilloma agents on test horses 37 and 38. This was true of both pre-exposure and postexposure serums. There was no difference in the size of the growths and in the time of their appearance which could be correlated with any neutralizing properties of these bovine serums.

Titrations of the infectivity of strain 200 indicated that horse 37 was more susceptible than horse 38. Strain 30 material was only slightly active in a 1:100 dilution on horse 37 and was completely inactive on horse 38.

No growths occurred on test horses 34 and 35 at the inoculation sites following the introduction of a mixture of either heated or unheated immune horse serum and papilloma agent.

Test horses 34 and 35 differed in their susceptibility to the wart preparations. A 1:10 dilution of wart material was active in both horses. Material diluted 1:50,000 was inactive in horse 35 while horse 34 developed growths from material diluted 1:100,000. Both heated and unheated nonimmune serum from horse 34 failed to neutralize the virus. However, while the nonimmune heated serum from horse 35 had no neutralizing activity the unheated serum had a slight but definite neutralizing capacity. When tested on horse 34, this serum neutralized the 1:10,000 virus dilution but failed to neutralize the 1:100 virus dilution. When tested on the more resistant horse 35, the unheated serum partially neutralized the 1:100 virus dilution as demonstrated by the comparative size and duration of the growths (fig. 2).

DISCUSSION

Serums from cattle previously exposed to papilloma virus failed to neutralize the latter in contrast with serums from immune horses (table 1). The cattle serums which did not neutralize the papilloma agent came from calves with either a few small growths or none at all while the horse serums with neutralizing capacity came from animals with a large number of growths. Since it seemed desirable to test the neutralizing activity of serum from a calf bearing a large number of well-developed warts, another experiment was

performed. Calf 75 was exposed to strains 30 and 207 of bovine papilloma material. There were about 50 relatively large warts three and one half months later when serum was taken to test its neutralizing ability. The neutralization trials were performed on the skin of test calves since no horses were available. The serum did not neutralize either strain in any of four dilutions.

The lack of *in vitro* neutralizing ability of serum from calves with papillomas is somewhat in contrast with the report of Post and Terpstra.[2] They gave rather large quantities of blood (100 to 1,500 ml.) obtained from a calf with warts to 3 test calves. At the same time, the 3 calves were experimentally exposed to active papilloma material. They did not develop warts, thus suggesting protective action of the blood.

Complete neutralization of papilloma virus by immune horse serums indicated that a circulating neutralizing antibody developed in horses after multiple intradermal exposure to bovine papilloma virus. This was correlated with the immunity of the horses to re-exposure. The neutralizing effect of the immune serum was not affected by heating at 56 C. for thirty minutes (table 1).

Normal pre-exposure serum from horse 35 had a slight but definite neutralizing effect which could be destroyed by heating at 56 C. for thirty minutes. This, and the fact that the animal had no history of papillomatosis, indicated that the neutralizing substance was not a specific antibody. Horse 35 was also more resistant to bovine papilloma virus than horse 34 (demonstrated by their susceptibility to different dilutions of the agent). Whether the degree of susceptibility of horses to the bovine papilloma agent is due to the heat-labile neutralizing serum factor, and whether this factor is similar to or identical with a protein, properdin, would be an interesting problem for further investiga-

TABLE 1—Neutralization of Bovine Papilloma Agent by Serums from Cattle and Horses Before (Nonimmune) and after (Immune) Experimental Infection with Bovine Papilloma Agent

Serum from		Treatment of serum	
		Unheated	Heated
Horses	Nonimmune	±	—
	Immune	+	+
Cattle	Nonimmune	—	....
	Immune	—	....

+ Neutralization; ± variable; — negative.

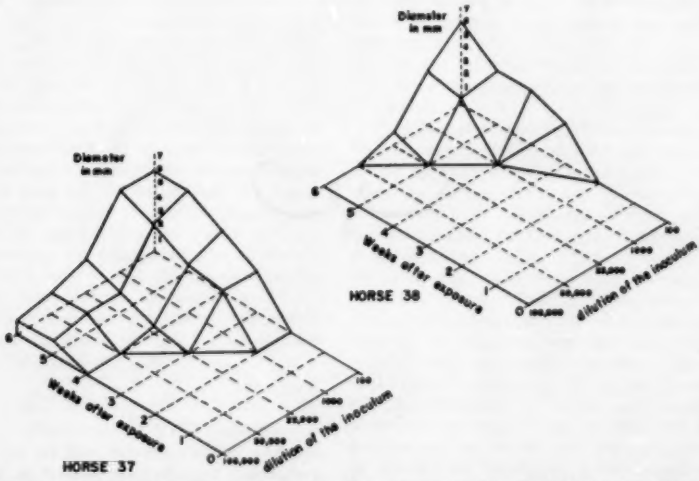

Fig. 3—Three dimensional graph of the rate of development of growths obtained on horses 37 and 38 following intradermal inoculation with different dilutions of bovine papilloma agent (strain 200). The size is given as the diameter in millimeters of the approximately hemispherical growths and represents the average of duplicate growths. Dilution expressed as 100 was actually 1 part tissue to 100 parts physiological salt solution. Note that the size of the growth is related to time as well as the dilution of inoculum. These relationships are similar in both the more susceptible horse 37 and relatively resistant horse 38.

tion. Properdin, a serum protein, has been shown responsible for the nonspecific resistance to infection of different species of animals.[5] Both the neutralizing effect of properdin in serum and the neutralizing effect shown by serum from horse 35 against papilloma virus are destroyed by heating at 56 C. for thirty minutes.

We have often observed that different horses respond differently to the same inoculum. An example of such variation in susceptibility to the bovine wart virus is illustrated (fig. 3). In addition to the variation in response of different animals of the same species, we have also observed differences in pathogenicity between strains of bovine papilloma agent. In these experiments, strain 200 was considerably more pathogenic for the horse than strain 30. These strains behaved in the opposite way in calves.[6] The same result of greater infectivity of strain 30 for calves was obtained in another experiment utilizing strains 30 and 200 after each had one laboratory passage in calves. We have no explanation for this observation.

CONCLUSION

1) Cattle with papillomas do not have neutralizing antibodies in their serums. Horses with growths caused by the agent of bovine papilloma do have neutralizing antibodies in their serum.

2) Horses vary in their susceptibility to bovine papilloma. A heat-labile neutralizing factor was found in the blood serum of a comparatively resistant horse.

3) A strain of bovine papilloma virus with low virulence for cattle was highly infective for the horse. Another strain with high virulence for cattle was of low infectivity for the horse.

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Study of the Properties of the Virus of Venezuelan Equine Encephalomyelitis Modified by *in Vitro* Cultivation in HeLa Cells

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IN A PREVIOUS paper, propagation of the virus of Venezuelan equine encephalomyelitis (VEE) in human malignant epithelial cells (strain HeLa) was reported.[1] It was shown that the virus of VEE could be propagated by serial passage in HeLa cell cultures producing cytopathogenic effects which could be prevented by the addition of immune serum.

The need for an attenuated VEE virus for immunization purposes became apparent when it was shown that VEE vaccine which passed rigid requirements of safety tests in animals produced infections in man.[2] Increasing the formaldehyde percentage for inactivation of the virus reduced the antigenic value of the vaccine.

Koprowski and Lennette,[3] employing a Maitland-type medium for culture of the virus of VEE, reported a marked reduction in pathogenicity by extraneural routes of inoculation. The question of the retention of antigenic properties and reversion to a virulent strain having properties of the original virus was discussed but not reported by them. They also reported on the influence of age on the susceptibility of mice to infection with VEE.[4] It was shown that mice of all ages tested (3 to 233 days) were about as susceptible to extraneural as to intracerebral inoculations. Because of this property and due to its simplicity, the intraperitoneal route has been used by our laboratory for many years for the performance of neutralization tests. This afforded an opportunity to follow the reduction in virulence by comparing the titers obtained with intracerebral and intraperitoneal injections of the same volume of culture fluid.

This paper describes the properties and reduction in pathogenicity obtained thus far by *in vitro* cultivation of the virus of VEE.

MATERIALS AND METHODS

Virus Strain.—The strain of VEE routinely employed in studies in this laboratory was isolated from the brain of a donkey which had succumbed to the disease during an epizootic in Trinidad, B.W.I., in October, 1943.[5] This strain has been through 16 mouse brain passages.

Infected mouse brains, representing the sixteenth passage, were ground in a mortar with alundum in 0.2 per cent bovine plasma albumin (BPA, Armour Laboratories) to yield a 10 per cent suspension by weight. This suspension was centrifuged at 16,000 r.p.m. for one hour in an international refrigerated centrifuge. The supernatant fluids were removed and used to initiate the culture series.

Tissue Culture.—A stable strain of malignant human epithelial cells, strain HeLa,* was used throughout this study.

The same procedures and techniques described in a previous report,[1] for the propagation of VEE for 21 passages in HeLa cells, have been continued throughout this study. The virus has been serially passed 65 times, producing cytopathogenic effects as previously described. Routine sterility tests were performed on each passage. Through 51 serial passages the cells were maintained in nutrient fluid containing 10 per cent human serum. From the fifty-second through the sixty-fifth passage the HeLa cells have been grown and maintained in mixture No. 199 containing 10 per cent horse serum.[6] At intervals during this study, neutralization tests were performed to verify the identity of the virus.

Test for Alteration of Pathogenicity for Mice.—The change in infectivity of the virus was determined by titration in mice employing, for comparison, the intracerebral and intraperitoneal routes of inoculation. All titrations were performed by the inoculation of the pooled supernatant fluids from infected HeLa tubes. Serial tenfold dilutions of the virus were prepared using 0.2 per cent BPA as a diluent. Unless otherwise stated, albino mice (Bagg strain) of mixed sexes, approximately 28 days of age and 12 to 14 Gm. in weight, were inoculated by the route of choice, each animal receiving 0.03 ml. of the appropriate dilution regardless of route of inoculation. The difference in infectivity, by the two routes, was determined by the 50 per cent end point method of Reed and Muench.[7] Calculations were made counting all deaths occurring between the second and tenth day in the case of animals receiving intracerebral inoculation and the second to fourteenth day in the case of intraperitoneal inoculation.

From the Veterinary Division, Army Medical Service Graduate School, Walter Reed Army Medical Center, Washington 12, D. C. Miss Blackford is now with the Naval Medical Research Institute, Bethesda, Md.

The authors gratefully acknowledge the technical assistance of Master Sergeant Clyde W. Boyd.

* From Microbiological Associates, Inc., Bethesda, Md.

Infectivity Tests in Rabbits.—Using two passages of the VEE-HeLa strain (20 and 60) rabbits were inoculated by various routes. The amount and dilution of the inoculum was arbitrarily chosen in the earlier experiment and this protocol was repeated for the second test. The twentieth HeLa passage titered $10^{-6.6}$ and the sixtieth passage, $10^{-4.9}$ intracerebrally in mice.

TABLE 1—Effect of Passage in HeLa Cell Tissue Culture on the Virulence of Venezuelan Equine Encephalomyelitis for Mice by the Intracerebral and Intraperitoneal Routes

Passage No.	Negative log of l.d.$_{50}$	
	Intracerebral	Intraperitoneal
0	9.2	....
5	>4.5	2.6
15	4.8	3.4
20	5.6	4.5
22	5.7	5.0
23	4.6	2.2
25	5.0	1.5
30	5.3	<1.0
35	5.3	<1.0
40	4.2	N
45	4.8	N
50	4.5	N
65	3.4	<1.0

N—no deaths with undiluted inoculum.

Rabbits, mixed sexes, weighing 2 to 3 kg. were used and their preinoculation temperatures were recorded. Four groups were inoculated using a 10^{-1} dilution of the desired passage as follows: intracerebral received 0.03 ml.; intraperitoneal, 0.10 ml.; intramuscular, 0.10 ml.; and subcutaneous, 0.05 ml. Temperatures were recorded daily and the animals were observed for symptoms of VEE infection for twenty-one days or until death.

Determination of Stability of Modified VEE Virus.—Using the forty-second HeLa passage which had a mouse lethal dose (l.d.$_{50}$) of $10^{-6.0}$ intracerebrally, mice were inoculated intracerebrally with 0.03 ml. of the undiluted supernatant fluid per animal. When the animals became moribund, they were anesthetized and brain tissue was removed aseptically. The tissues were ground in a mortar and suspended in 0.2 per cent BPA to yield a 10 per cent suspension by weight. The material was centrifuged at 16,000 r.p.m. in an international centrifuge and the supernatant fluid decanted. Five passages were made intracerebrally in mice and the fifth passage titrated both intracerebrally and intraperitoneally as described above.

Again using the forty-second HeLa passage, 8-day-old embryonating White Leghorn eggs were inoculated via the allantoic cavity with 0.10 ml. of the undiluted supernatant fluid per embryo. Eggs were incubated at 37.5 C. and candled twice daily. Dead embryos were chilled overnight and the allantoic fluid harvested and passed, undiluted, to a new group of eggs. Five egg passages were made and the fifth passage was checked for infectivity as before.

Potency Test in Guinea Pigs.—Potency tests were performed on the thirty-sixth, fiftieth, and sixty-fifth HeLa passages. The l.d.$_{50}$ titers intracerebrally in mice for these passages are as follows: thirty-sixth, $10^{-3.7}$; fiftieth, $10^{-4.5}$; and sixty-fifth, $10^{-3.4}$. In each test, 6 guinea pigs weighing 350 to 450 Gm. were inoculated intradermally with one dose of 0.1 ml. of the undiluted supernatant fluid of the desired passage. On the fifteenth post-vaccination day all animals were challenged by intracerebral inoculation with 0.1 ml. of a guinea pig brain suspension of VEE (Trinidad strain). This strain gave an l.d.$_{50}$ in guinea pigs by the intracerebral route of $10^{-5.0}$. The vaccinated animals were divided into two groups, one group receiving 10,000 infective doses and the other, 1,000 infective doses. Two unvaccinated control groups received the same challenge doses. Temperatures were recorded daily on all groups during a fourteen-day observation period.

RESULTS

Alteration of Pathogenicity for Mice.—Various passage levels of the HeLa cell-adapted VEE virus were titrated in mice by the intracerebral and intraperitoneal routes. A comparison of the l.d.$_{50}$ titers of the two routes shows that although the titer of the HeLa-adapted virus is lower than the mouse brain strain, it was similar for each passage tested by the intracerebral route. Beginning with the twenty-third passage, it was evident that the pathogenicity of the virus was decreasing when inoculated intraperitoneally. By the twenty-fifth passage, the virulence had markedly decreased and this change has persisted through 65 passages (table 1).

Effect of Age on Susceptibility of Mice to the Modified VEE Virus.—The change in pathogenicity by the intraperitoneal

TABLE 2—Effect of Age on Susceptibility of Mice to the Modified Venezuelan Equine Encephalomyelitis Virus

Passage No.	Age of mice (days)	Negative log of l.d.$_{50}$	
		Intracerebral	Intraperitoneal
40	1	6.18	4.60
40	7	5.55	4.34
40	14	4.50	4.50
40	21	4.10	1.75
40	28	4.20	N
50	14	4.63	4.0
50	28	4.5	N
61	14	4.23	2.35
65	14	3.40	1.10
65	28	3.4	<1.0

N—no deaths with undiluted inoculum.

route in mice was demonstrated, using mice (12–14 Gm.) approximately 28 days old. To determine if this alteration in virulence was evident in younger animals, groups of mice 1, 7, 14, and 21 days old were inoculated as before with the fortieth HeLa passage. With this passage, there was no evidence that the pathogenicity of the virus for mice 1 to 14 days of age had been affected; however, mice 21 days of age were more refractive when inoculated intraperitoneally. Subsequent titrations using later passages of the virus and 14-day-old mice indicated that younger animals were becoming less susceptible to the virus by the intraperitoneal route (table 2).

Infectivity for Rabbits.—Rabbits are highly susceptible to VEE virus by all routes of inoculation. To determine if this virus had been modified for rabbits as well as for mice, rabbits were inoculated via the intracerebral, intraperitoneal, subcutaneous, and intramuscular routes with the twentieth and sixtieth HeLa passages as the inoculums (table 3).

Three of 4 rabbits receiving the twentieth HeLa passage succumbed to viral infection. All 4 animals demonstrated a significant increase in temperature. The rabbit receiving the intraperitoneal inoculation survived the twenty-one-day observation period and was challenged by intracerebral inoculation with 0.05 ml. of a 10^{-2} suspension of VEE–MB No. 10 (Trinidad) which titered $10^{-6.8}$ intracerebrally in mice. The animal withstood the challenge without temperature elevation or clinical symptoms and was destroyed in good health, six weeks later.

Only 1 of 8 rabbits which received the sixtieth HeLa passage by various routes of inoculation died. Three of the 8, 2 receiving the intracerebral inoculation and 1 the intramuscular, demonstrated a significant elevation of temperature.

TABLE 3—Route, Dosage, and Alteration of Pathogenicity of Venezuelan Equine Encephalomyelitis (HeLa Strain) for Rabbits

Route of inoculation	Dose (ml.)	Mortality ratio	
		HeLa 20*	HeLa 60**
Intracerebral	0.03	1/1	1/2
Intraperitoneal	0.10	0/1	0/2
Subcutaneous	0.05	1/1	0/2
Intramuscular	0.10	1/1	0/2

*Intracebral titer in mice $10^{-5.6}$
** Intracerebral titer in mice $10^{-4.9}$

The data indicate that after prolonged cultivation in HeLa cells a marked reduction in pathogenicity for the rabbit has taken place.

Stability of Modified VEE Virus.—Data indicate that the passage of the VEE virus in HeLa cells has reduced its virulence for mice by the intraperitoneal route. It was of interest to determine whether a reversion to the original virulent strain would occur if the HeLa modified strain were passaged several times in mice and in chicken embryos.

TABLE 4—Results of Potency Test in Guinea Pigs Immunized with Various Passages of Attenuated Venezuelan Equine Encephalomyelitis Virus

Immunizing agent		Results of challenge			
		Mortality ratios			
		Immunized		Nonimmune controls	
HeLa passage No.	$l.d._{50}$ in mice intracerebrally	1,000 i.d.*	10,000 i.d.	1,000 i.d.	10,000 i.d.
36	$10^{-3.7}$	1/6	0/6	3/3	3/3
50	$10^{-4.5}$	0/6	0/6	3/3	3/3
65	$10^{-3.4}$	0/6	0/6	3/3	3/3

* Infective doses.

Brain suspensions of the fifth passage in mice titrated $10^{-5.3}$ intracerebrally in mice, whereas the titer by the intraperitoneal route was $<10^{-1.0}$.

Pooled allantoic fluids from the fifth egg passage were titrated in mice as mentioned above. The $l.d._{50}$ titer by the intracerebral route was $>10^{-7.0}$ and by the intraperitoneal $10^{-2.8}$.

The results show that after five passages through mice there is apparently no reversion to the original virulent form. On the other hand, passage through chicken embryos increased the virulence of the virus by both routes of inoculation.

Immunization of Guinea Pigs.—The group of 12 guinea pigs vaccinated with the thirty-sixth HeLa passage showed an elevation in temperature from the second to the sixth postinoculation day. The 12 which received the fiftieth HeLa passage demonstrated a slight temperature rise from the first to the third postinoculation day, except that the temperature of 1 remained elevated until its death on the tenth day. Using the sixty-fifth passage as a vaccine, none of the 12 animals showed a significant rise in temperature during the observation period.

All 36 animals challenged fifteen days after inoculation withstood the challenge except 1 which had received 1,000 l.d.$_{50}$ infective doses of the thirty-sixth HeLa passage. None of the other animals demonstrated an elevation in temperature or any evidence of illness during the observation period whereas all 18 control animals succumbed between the third and fifth day after inoculation (table 4).

A study of the antigenic properties of the HeLa modified virus has shown that after several passages through a foreign host, *i.e.*, HeLa cells, the living virus confers a good immunity on guinea pigs with only slight evidence of infection.

SUMMARY AND CONCLUSIONS

1) The virus of Venezuelan equine encephalomyelitis (VEE) has been serially propagated through 65 HeLa cell passages.

2) Beginning with the twenty-third passage, an alteration of pathogenicity for mice occurred. From the thirtieth to the sixty-fifth HeLa passage, mice approximately 28 days of age were refractive to the attenuated VEE virus when administered intraperitoneally at dilutions of 10^{-1} or less but were susceptible when inoculated intracerebrally.

3) Mice from 1 to 14 days of age were as susceptible to the intraperitoneal route as to the intracerebral route when inoculated with the fortieth HeLa passage. However, when given intraperitoneally the virulence was reduced for mice 21 days of age by the fortieth passage and for mice 14 days of age by the sixty-first and sixty-fifth passage.

4) Three of 4 rabbits died as the result of inoculation by four routes of the twentieth HeLa passage whereas only 1 of 8 died after the sixtieth passage.

5) After five intracerebral passages in mice of the forty-second HeLa passage modified virus, it was still avirulent for mice by the intraperitoneal route. Passage of the same strain five times in the allantoic cavity of eggs increased the infectivity for mice for both the intracerebral and intraperitoneal routes.

6) Guinea pigs immunized intradermally with one dose of various passage levels of live HeLa modified VEE virus withstood a challenge of 1,000 and 10,000 infective doses of a virulent strain of VEE virus.

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Bacterial Permeation of the Gut Wall in Irradiated Burros

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ONE OF THE factors involved in the radiation syndrome is the transfer of bacteria through the normal body barriers of an animal. The belief is held by some that a bacteremia is largely responsible for the death of irradiated animals.[14]

It has been found that even avirulent bacteria [9, 28] invade the body tissues of an animal irradiated with x-rays [13, 14] or *gamma* rays [11, 25] and that resistance to infection is lowered.[3, 17, 19] The immune response of the animal is varied [7, 12, 20] and may be either beneficial or deleterious. Leukopenia of radiation [6, 19] may be a factor contributing to the lowered resistances of the animal.

Warren and Whipple [24] observed toxemia and bacteremia in irradiated dogs and found a correlation between the bacteremia and destruction of the epithelium of the intestine. The invasion was not as rapid as they expected, probably because there was an outflow of liquid into the intestine, thereby washing the bacteria away from the intestinal wall. Chrom [4] observed, by staining a section of the intestine, that bacteria apparently adhere to the intestinal wall of irradiated mice but did not observe this phenomenon with normal animals. Bacteria in blood were not observed immediately after irradiation but appeared about forty-eight hours later.[12]

Our investigation was designed to demonstrate the invasion of microorganisms through the intestinal wall and to correlate this invasion with dose rate and the appearance of pathological changes associated with the radiation syndrome in the burro.

MATERIALS AND METHODS

The animals used in this experiment were randomly selected female burros, *Equus asinus asinus*. The choice of this species was influenced by the data available concerning the behavior of the burro following exposure to total body ionizing radiation [8, 15, 16, 22] and the facility with which these animals would lend themselves to necessary surgical procedures and sampling manipulations. Except during daily periods of irradiation, the animals were stabled in partially covered and bedded holding pens.

Exposures were made on the radiation field of the University of Tennessee-Atomic Energy Commission described by Wilding, Simons, and Rust.[25] Each animal wandered at will within the confines of the exposure field and received total body irradiation from a constant flux of *gamma* rays from cobalt-60 sources. The rate was 40 r per hour. One group of 4 animals received 25 r per day; another group of 2 animals received 100 r per day; a third group of 3 animals received 400 r per day. In each case, daily irradiation was continued until exitus and for each group a non-irradiated control animal was maintained and manipulated in a manner identical to that of the test animals.

The animals which received 25 r per day were examined for the presence of microorganisms in the blood and bone marrow, and after death were examined for the presence of microorganisms in various internal organs. In animals which received 100 r and 400 r per day, examination was made for the passage of a tracer organism through the intestinal wall, both *in vivo* and by means of an *in vitro* preparation of excised gut. In addition, studies were made to determine the presence of the tracer organism in blood, bone marrow, and in internal organs.

Daily samples of blood were obtained aseptically by jugular venipuncture. Bone marrow was obtained every second day from a puncture of tuber coxae or lumbar vertebra. Samples of liver, lung, spleen, brain, adrenal glands, kidney, and synovial fluid were aseptically obtained immediately after death. Samples were similarly taken from control animals.

Bacterial counts were made of blood or serum and bone marrow on trypticase soy agar plates. The cultures were made in duplicate and incubated at 37 C. for one week. Bacterial counts of synovial fluid were obtained by spraying and incubating the sample on plates of hardened trypticase soy agar. Two methods were used to determine the presence of microorganisms in the organ samples. In some cases, impressions of the tissues were made directly to trypticase soy agar plates; other samples were first ground in a Waring blendor with sterile water before plates were inoculated.

One unique facet of this experiment involved the use of an artificial fistula into the cecum of the animal into which a tracer organism was injected before and during the period of irradiation. This was done with test and control animals in the 100 r and 400 r groups. The surgical procedure used in the production of the fistulas was an adaptation of that described by Alexander *et al.*[1] and by Jasper and Cupps.[10] The two-stage operation consisted of exteriorizing the base of the cecum in the pocket of the right flank and subse-

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quently incising this organ. The resultant fistula was large enough to permit insertion of a 10-ml. pipette and at the same time small enough that muscle tonus of the flank and a slight swelling of the cecal mucous membrane effectively closed the opening from the outside (fig. 1).

The tracer organism employed to detect bacterial transfer was *Serratia marcescens*. The typical red pigment produced by this organism is easily distinguishable upon cultivation. Though not a normal inhabitant in this animal, the organism was found to survive in the burro's intestinal tract. The bacteria were grown on trypticase soy agar in Blake bottles. A heavy suspension in physiological saline (5.0×10^9 cells/ml.) was prepared for daily inoculations into the cecum. Following the withdrawal of a 10-ml. sample of cecal content, 10 ml. of the suspension was introduced by means of a pipette.

In an attempt to demonstrate the passage of *S. marcescens* through the isolated intestinal wall, a section of the intestine was taken from each animal during necropsy, washed with sterile physiological saline, and suspended in a 1-liter Erlenmeyer flask with a plugged glass tube inserted through the rubber stopper. The tube was inserted into one end of the intestinal section, and

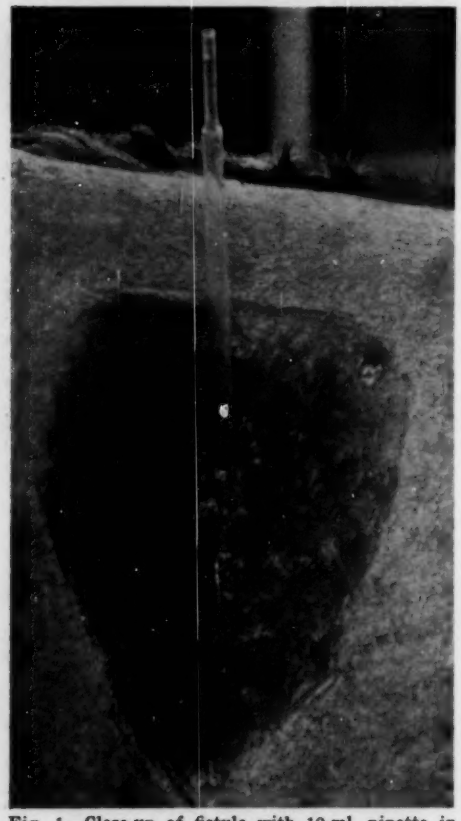

Fig. 1—Close-up of fistula with 10-ml. pipette in position to withdraw intestinal sample.

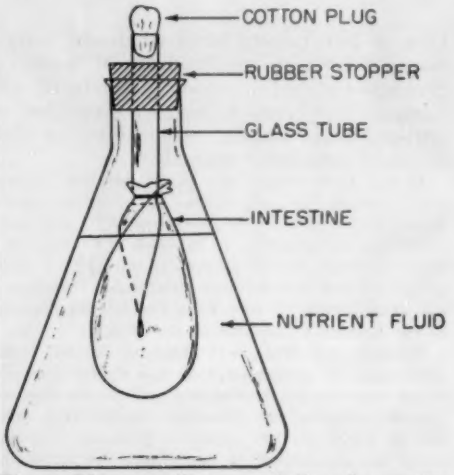

Fig. 2—Diagrammatic representation of apparatus used to measure in vitro permeability of irradiated burro intestine.

the other end was closed with surgical thread and tied to the tube. This formed a loop which was suspended in the nutrient broth in the flask. A suspension of the organism in nutrient broth was placed in the intestine through the glass tube (fig. 2). At regular intervals, a sample of the broth was withdrawn from the flask and plated on trypticase soy agar. The plates were incubated at room temperature and examined for colonies of the tracer organism.

RESULTS

The normal bacterial flora of the intestinal tract of the burro contained species of Proteus, Bacillus, Streptomyces, Streptococcus, Pseudomonas, Alcaligenes, and coliforms.

Proteus spp. and aerobic sporeformers were found in many organs following irradiation (table 1). Gram-negative rods which fermented lactose were demonstrated in lesser numbers. *Streptomyces coelicolor* or a closely related Streptomyces organism was found in several organs and the blood. Pseudomonas organisms were isolated from liver, kidney, and lungs; Alcaligenes were isolated from liver, spleen, and synovial fluid. *Streptococcus fecalis*

TABLE 1—Postmortem Isolations of Various Micro-organisms from Burro Tissue Following Lethal Total Body Irradiation at 25 r Per Day

Organism	Tissues									
	Blood	Bone marrow	Liver	Synovial fluid	Spleen	Adrenals	Kidney	Brain	Lung	Intestine
Proteus	+	—	+	+	+	—	+	—	+	+
Bacillus	+	+	+	+	+	+	+	+	—	+
Coliforms	+	—	+	—	+	+	—	+	+	+
Streptomyces	+	+	+	—	+	—	+	—	—	+
Sarcina	—	—	—	—	—	—	—	+	—	—
Str. faecalis	—	—	+	—	+	—	+	—	—	—
Pseudomonas	—	—	+	—	—	—	+	—	+	—
Alcaligenes	—	—	+	+	+	—	—	—	—	+

+ Organisms present; — organisms absent.

was found in liver, spleen, and kidney. Sarcina, not found in the intestinal tract of burros examined, was isolated from one brain. None of these organisms was isolated from normal, nonirradiated burros except from the intestinal tract.

During the period of irradiation *Serratia marcescens* introduced into the intestinal tract of burros was recovered on only one occasion. The blood of an animal exposed at 400 r per day and, on two occasions, the bone marrow of an animal exposed at the rate of 100 r per day yielded *S. marcescens.* Recoveries of the organism from the intestinal content of irradiated and control animals were made routinely. After death of the animals, *S. marcescens* was recovered from various internal organs; in the animals exposed to 100 r per day, it was recovered from the spleen, kidney, and liver of 1 animal and from the kidney and lung of the other animal; in the group exposed at 400 r per day, the tracer organism was recovered from the kidney of 1 animal.

As shown in table 2, utilizing the *in vitro* intestine preparation described, *S. marcescens* permeated the gut of both animals irradiated at 100 r per day, but no recovery was made from those irradiated at 400 r per day or from the respective control animals.

The animals exposed at the rate of 100 r per day survived twenty-two days. Two of the animals exposed to 400 r per day survived nine days and 1 animal seven days.

Discussion

Microorganisms were found to pass from the intestine into the blood stream and internal organs of burros subjected to total body *gamma* irradiation as observed by others in laboratory animals.[13,14] The tracer organism, *S. marcescens* similarly found in blood and other tissue after inoculation into the gut, has been reported able to pass through the normal intestinal wall, but this phenomenon is believed to be a rare occurrence.[2] There was no evidence of this in our control animals.

The variation in the incidence of recoveries of organisms in groups subjected to different dose rates merits consideration. The tracer organism, *S. marcescens,* was recovered with relatively more frequency from the animals receiving 100 r per day than from those receiving 400 r per day. This was again demonstrated in the *in vitro* studies using excised gut from irradiated and nonirradiated animals of the two groups. The relative degree of injury manifested in the gut may be a function of survival time. A hemorrhagic phase in the radiation syndrome[6] is not observed in burros having received a dose sufficiently large to cause an early death.[21] Accordingly, in the case of the group receiving 100 r per day, both animals presented evi-

TABLE 2—In Vitro Intestinal Transfer of Serratia Marcescens

Hours after inoculation	100 r group by number			400 r group by number			
	48	139	135 (control)	50	102	222	168 (control)
6	—	—	—	—	—	—	—
12	1.3×10^3	9.2×10^3	—	—	—	—	—
24	3.2×10^4	9.0×10^4	—	—	—	—	—
48	2.4×10^6	1.2×10^5	—	—	—	—	—
62	1.2×10^7	1.3×10^8	—	—	—	—	—

— Organisms absent.

dence of characteristic hemorrhagic symptoms clinically and on postmortem examination; whereas, the group irradiated at 400 r per day did not. Therefore, it may be that bacterial transfer and the appearance of the hemorrhagic diathesis are concurrent physiological changes associated with survival time.

SUMMARY

The bacterial invasion from the gut was observed in western burros *(Equus asinus asinus)* given total body irradiation at the rates of 25, 100, and 400 r per day until death.

After irradiation at 25 r per day, microorganisms commonly found in the intestines of the experimental animal were found in the blood and internal organs. These organisms were primarily Proteus and aerobic sporeformers and, to a lesser extent, coliforms, Pseudomonas, Alcaligenes, and a Streptomyces resembling *Streptomyces coelicolor.*

The organism, *Serratia marcescens,* inoculated into the cecum of burros receiving 100 r per day, was found in the blood and internal organs. *In vitro* this organism was also able to traverse the intestinal wall of irradiated burros but not of nonirradiated burros.

In vivo and *in vitro* studies indicate that in burros irradiated at the rate of 400 r per day, changes in the intestinal wall permitting microorganisms to pass in great numbers had not occurred before death at nine days.

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Some Characteristics of Transmissible Gastroenteritis (TGE) in Disease-Free Antibody-Devoid Pigs

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SINCE the first report by Doyle and Hutchings [1] in 1946 of transmissible gastroenteritis (TGE) as a transmissible disease of baby pigs, few papers dealing with quantitative aspects of the infection and the infectious agent have been published. The most comprehensive study was recently reported by Lee, Moro, and Baker,[2] using as the test host, pigs which had nursed their dams. The purpose of this paper is to present data obtained with TGE in disease-free antibody-devoid pigs procured by hysterectomy [3] and housed in individual isolation units.[4] Such pigs were used because of their uniformity in response following inoculation with infectious agents. Variation of results with a specific agent due to simultaneous presence of a variety of secondary organisms is minimized.

MATERIALS AND METHODS

Experimental Pigs.—All pigs used in these studies were outbred Chester White pigs procured from their dams two to four days prematurely by hysterectomy.[3] They were housed from birth, without exposure to airborne infectious agents, in individual virus isolation units.[4] They were fed, twice daily, a ration consisting of pasteurized homogenized cows' milk, whole egg, and inorganic salts [4] at a quantity of one-sixth quart per pig per feeding. Room temperature in which the isolation units were kept was maintained at 85–90 F. Unless otherwise specified, all pigs were used for inoculation five days after procurement.

TGE Virus.—The virus used throughout these studies was obtained from the lung of a 6-day-old pig at death during a 1949 epizootic within the herd of the Hormel Foundation. It has since been passed 14 times in young pigs by intranasal instillation of a 10^{-2} dilution of supernatants from minced lung tissue suspensions.

A standard suspension of TGE virus was prepared for these studies by inoculation of 2 pigs with the fourteenth passage virus handled as follows: The pigs were killed at the height of the signs forty-eight hours after inoculation. Their intestines, with their highly fluid contents, were removed to a sterile Waring blendor bowl to which was added approximately nine parts of cold buffered physiological saline. These materials were blended for 1.5 minutes under a bacteriological hood equipped with ultra-violet sterilizing lamps. The bowl was then chilled and the material blended for another 1.5 minutes. The coarse particulate matter was spun down at 1,000 times gravity for thirty minutes at 5 C., and the supernatant was decanted. The supernatant was recentrifuged at 5 C. and 10,000 times gravity for thirty minutes to reduce contaminating bacteria. The supernatant from this second centrifugation, which involved several separate runs because of the volume involved, was pooled and treated at room temperature for one to two hours with 1,000 units of penicillin and 1 μg. of streptomycin per milliliter of suspension. The material was then distributed to small glass vials, sealed, quick-frozen in an acetone-dry ice bath, and stored at −20 C. until used. This standard suspension of virus, on titration in 5-day-old pigs by intranasal instillation, would cause typical TGE infections routinely at dilutions of 10^{-6} but only rarely at 10^{-7}. After storage for six months at −20 C., the titer of the standard suspension remained 10^{-6}. This virus is quite stable as its infectivity titer drops approximately one log each twenty-four hours on exposure to 37 C. temperatures.

Virus Titration.—Titrations for TGE virus concentration in a variety of tissues were made by a standard procedure. Tissue donor pigs were inoculated intranasally with the standard TGE virus suspension and were killed under chloroform anaesthesia at specified intervals after inoculation. A blood sample was taken aseptically from the anterior vena cava and transferred directly to ampoules. Portions of nasal mucosa, lung, cardiac and pyloric portions of the stomach, duodenum, jejunum, ileocecal pouch, kidney, liver, testis, and spleen were removed with individual sterile instruments and placed in sterile Petri dishes. Tissues least apt to contain virus were taken first to minimize virus contamination.

Portions of tissue removed as above were weighed and then placed in a Waring blendor microbowl. Sufficient cold hormone broth was added to make a 10 per cent tissue suspension. The tissue and broth were then mixed for two 1.5-minute periods. The coarse particles were removed by low-speed centrifugation and the super-

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natant was decanted to other centrifuge tubes. The supernatant was then centrifuged at 5 C. at 10,000 times gravity for 1 hour. Three 2-ml. aliquots were carefully removed from the center of the centrifuge tube and sealed in glass ampoules. The material was quick-frozen by immersion in an acetone-dry ice bath and stored at —20 C. until titrated.

To titrate the 10 per cent tissue suspensions for content of TGE virus, one of the triplicate samples prepared was allowed to thaw at room temperature. Tenfold dilutions of the suspension were made in hormone broth. One milliliter of each dilution in a range expected to be infective was instilled into the nostrils of one 5-day-old pig. Pigs were observed twice daily for ninety-six hours for evidence of vomition and diarrhea. Absence of these signs beyond seventy-two hours after inoculation with dilutions of TGE virus was a clear indication that insufficient virus was present to cause infection. Inapparent TGE infections with resulting immunity occurred rarely, if at all, in 5-day-old disease-free antibody-devoid pigs. Under such conditions, TGE is an all-or-none disease.

End points on TGE infections are defined as the highest dilution of a suspension capable of eliciting disease. In every case in these studies, end points were established only when there was one or more higher dilutions given pigs which did not elicit disease. When the highest dilution tested contained sufficient virus to cause disease, a retest was made extending the dilutions beyond the infectivity end point.

Inoculation of Pigs.—The standard suspension of TGE virus was used at different dilutions to inoculate 5-day-old pigs. Intranasal, intratracheal, intragastric, and intraperitoneal inoculations were made to determine the relative susceptibility by different routes of inoculation. Intranasal inoculations were made by instilling 0.5 ml. of the given dilution of TGE suspension into each nostril of the host pig while under light ether anaesthesia. Intratracheal inoculations were also made with the pig under anaesthesia. The skin was incised just cephalad to and over the point where the trachea enters the thorax. One milliliter of TGE virus suspension was injected on inspiration into the exposed trachea through a 24-gauge hypodermic needle inserted between adjacent tracheal rings. Intragastric instillation was made through a 6-mm. rubber tube after it was passed to the stomach. One milliliter of TGE virus suspension was followed by two 5-ml. rinsings with hormone broth. The tube was withdrawn slowly to minimize chance of contaminating the respiratory tract. Intraperitoneal injections were made in the usual manner.

Specific TGE Antibody.—Two 16-week-old experimental pigs were injected with TGE virus to stimulate production of specific TGE antibody for *in vivo* and *in vitro* neutralization tests. The pigs were first given 10^6 infectious doses (i.d.) of TGE virus orally. This was followed by two injections of 3×10^6 oral i.d. of virus intramuscularly fourteen and twenty-eight days, respectively, after the oral inoculation. Both pigs were bled out twenty-one days after the last injection. Serum was harvested, pooled, and tested for presence of specific serum neutralization TGE antibody as previously described.[5]

Virus Filtration.—Gradacol membranes* were used in studies designed to estimate the particle size of TGE virus. The standard TGE virus suspension described was twice washed by centrifugation at 15,000 times gravity for one hour followed by resuspension in hormone broth, recentrifugation, and resuspension.** The final suspension was made so that each milliliter contained approximately 10^4 intranasal i.d. of TGE virus.

An attempt was made to pass the washed TGE suspension through gradacol membranes at 15 cm. of mercury vacuum. The membranes had been thoroughly washed with distilled water and steamed in an autoclave for one hour. The same membranes were used for replications, with thorough though gentle washing and resterilization between experiments. One milliliter of the fluid which passed the membranes was instilled into the nostrils of 5-day-old pigs. An individual pig was used for each filtrate.

TABLE 1—Estimation of Size of Virus of Transmissible Gastroenteritis by Means of Gradacol Membranes

Replication	Pore diameter size in millimicrons			
	120	170	230	420
1	—	—	+	+
2	—	—	+	+
3	NT	—	+	NT

— Host remained normal; + host evidenced typical TGE; NT not tested.

PROCEDURES AND RESULTS

Estimation of Particle Size of TGE Virus.—From the data obtained in three replicate experiments (table 1), it is apparent that TGE virus passes membrane pores 230 mμ in size but not 170 mμ in size. Particle size of the infectious agent, therefore, is estimated at approximately 200 mμ.

Susceptibility of Pigs to TGE by Different Routes of Inoculation.—The results obtained on inoculation of 5-day-old pigs with different concentrations of TGE virus and by different routes (table 2) indicate quite clearly that the tissues of the gastrointestinal (GI) tract are most easily attacked by TGE virus. Approximately 90 per cent of material introduced intranasally goes to the GI tract. Since the lung of 5-day-old pigs is seemingly refractory to TGE, the high infectivity and lethality of TGE following intranasal instilla-

* We are indebted to Dr. S. E. Luria, Department of Bacteriology, University of Illinois, for supplying the membranes.

** Carefully removed supernatants of TGE virus suspensions centrifuged 15,000 times gravity for one hour are noninfective.

tion must be due to GI infection or, conceivably, infection of upper respiratory tissues and the GI tract simultaneously.

Transmissible gastroenteritis infection with 10^5 i.d. given intratracheally, when lesser doses failed to cause infection, could have resulted from GI infection from a portion of the large inoculum of TGE spilling over into the esophagus. At least very large doses of TGE virus are required to cause infection on intratracheal instillation. This was also true relative to intraperitoneal inoculations.

Relationship of Host Age to Site of Virus Multiplication and Course of TGE Infection.—To define the relationship of age of host to infection with TGE, three experiments were carried out with pigs of different ages. The first was with 2-hour-old pigs. Five pigs were each given approximately 10 i.d. of TGE virus intranasally, then returned to isolation. These pigs were killed four, eight, twelve, fourteen, and sixteen hours, respectively, after inoculation and their tissues were handled as described under "Materials and Methods" for quantitative titration for TGE virus (table 3).

The rapidity with which TGE virus multiplies in the newborn pig is emphasized by a definite increase of TGE virus in lung, duodenum, and jejunum of the pig killed eight hours after inoculation. The positive finding in duodenum diluted 1:10 in the pig killed four hours after inoculation may have resulted from residual virus from the inoculum. Findings in pigs killed twelve, fourteen, and sixteen hours after inoculation were essentially the same as those with the pig killed at eight hours.

In a second experiment (table 4), four 6-day-old pigs were inoculated with approximately 100 i.d. of TGE virus and returned to isolation. One pig was killed twelve hours later, the second twenty-four hours later, the third and fourth pigs forty-eight and seventy-two hours later. Tissues were titrated as was previously done with tissues from pigs inoculated when two hours old.

The striking difference between the 2-hour and 6-day-old pigs was in the response of upper respiratory and respiratory tissues to TGE virus. Whereas the pigs inoculated when 2 hours old had refractory upper respiratory tissues but susceptible lung tissues, the exact opposite situation was presented in pigs inoculated when 6 days old. The further finding of relatively low titers of TGE virus in nonepithelial tissues and blood in the latter group of pigs is thought to be associated primarily with viremia and accumulation of virus by filtration or local absorption.

In a third experiment (table 5), 12 pigs were used to evaluate the increase in resistance of pigs with age (maturation resistance). The pigs were raised in three groups of 4 each in isolation units. When these groups were 11, 17, and 24 days old, respectively, they were inoculated with TGE virus, 1 to 100 intranasal i.d., for 5-day-old pigs. This dosage of TGE represented 10^{-6}, 10^{-5}, and 10^{-4} dilutions of the standard TGE suspension. The pigs were observed for eight days for evidence of TGE infection.

All 12 pigs became infected with TGE although

TABLE 2—Relative Infectivity of Virus of Transmissible Gastroenteritis (TGE) by Different Routes of Inoculation in 5-Day-Old Pigs

Route of inoculation	Dilution of standard TGE suspension*	Manifestation of signs (vomition, V, and diarrhea, D) in hours after inoculation													
		24 hr.		48 hr.		72 hr.		96 hr.		120 hr.		144 hr.		168 hr.	
		V	D	V	D	V	D	V	D	V	D	V	D	V	D
Intranasal	10^{-1}	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	10^{-2}	—	—	—	—	—	+	—	+	—	+	—	+	—	+
	10^{-3}	—	—	+	+	+	+	Dead							
	10^{-4}	+	+	+	+	+	+	Dead							
	10^{-5}	+	+	+	+	+	+	Dead							
	10^{-6}	+	+	+	+	Dead									
Intratracheal	10^{-3}	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	10^{-4}	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	10^{-5}	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	10^{-6}	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	10^{-7}	—	—	—	—	+	+	+	+	+	+	+	+	+	+
Intragastric	10^{-1}	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	10^{-2}	—	—	—	—	—	—	+	+	+	+	+	+	—	+
	10^{-3}	—	+	—	—	—	—	—	—	—	—	—	—	—	—
	10^{-4}	+	—	+	+	+	+	+	+	+	+	+	+	+	+
	10^{-5}	+	+	+	+	Dead									
	10^{-6}	+	+	+	+	Dead									
Intraperitoneal	10^{-6}	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	10^{-5}	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	10^{-4}	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	10^{-3}	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	10^{-2}	—	—	—	—	—	+	Dead							

* Suspension diluted 10^{-6} contains approximately one infectious dose (i.d.) of TGE virus, 10^{-5} contains 10 i.d., 10^{-4} contains 100 i.d., etc.; — animal normal; + signs manifested.

there were striking differences in the severity of the disease which must be attributed to age factors. The 11-day-old pigs were most severely infected. One of the 4 pigs died on the fourth day; all had vomited within twenty-four hours, and all but 1 had manifested diarrhea by that time. Diarrhea persisted in the 3 living pigs through the eight days of observation.

A more moderate TGE infection was evident in the 17-day-old pigs. One of the 4 pigs never vomited, while another manifested diarrhea the third and fourth day, then returned to normal. The diarrhea was less watery and discharges were less frequent than in the 11-day-old group.

In the 24-day-old pigs TGE had to be demonstrated indirectly except for a single vomition in 1 pig twenty-four hours after inoculation. Blood samples were taken from the anterior vena cava of each pig at each 24-hour interval, placed in ampoules, sealed, quick-frozen in a dry ice bath and stored at —20 C. These samples were later tested for TGE virus by intranasal inoculation of the material into 5-day-old pigs. Each of the 4 pigs had demonstrable viremia within five days of inoculation. The pig receiving the least virus, *i.e.*, the 10^{-6} dilution of the standard suspension, was the first by twenty-four hours to have demonstrable viremia. Two others had viremia in seventy-two hours while infected blood was not obtained from the fourth pig until the 120-hour sample.

Demonstration of Specific TGE Antibody.—Serum from two sources was tested for presence of serum neutralizing TGE antibody. One source was 3 disease-free antibody-devoid pigs which were inoculated intranasally at 14 days of age with TGE virus. These pigs were killed fifteen days later and their serums tested for the presence of serum neutralizing antibody. Serums obtained from the same pigs just before inoculation with TGE virus were tested simultaneously (table 6). It is apparent that demonstrable SN antibody in 2-week-old pigs was not present in either the preinoculation or postinoculation serums.

A similar test was applied to serums from 2 pigs inoculated when 16 weeks old. Details of preparation of these serums have been given previously. The data (table 6) indicate that SN antibodies for TGE virus were present in the serums obtained several weeks after inoculation with TGE virus. There was no indication of SN activity in the preinoculation serums.

Further tests were made on serums obtained from the 2 pigs inoculated with TGE virus at 16 weeks (table 7). Tests on combined serums indicate that serum diluted 1:32 contains sufficient SN antibody to neutralize 1,000 intranasal i.d. of TGE virus. Serum diluted 1:64 was incapable of neutralizing 1,000 i.d. of TGE virus.

A test was also made with the specific anti-TGE serum obtained from the older pigs for its ability to protect the host against active TGE infection. Eight pigs from a single litter were treated as follows: (1) Two pigs were left as normal uninoculated controls; (2) 2 pigs were inoculated with 1,000 i.d. of TGE virus when twenty-four hours old to serve as virus controls; (3) 2 pigs were given 50 ml. each of the anti-TGE serum by stomach tube when four hours old. They were inoculated with TGE virus when 24 hours old, and (4) 2 pigs were each given 20 ml. of the serum intraperitoneally in 10-ml. doses twenty and twenty-four hours, respectively, after birth. The second serum injection was accompanied by the standard 1,000 intranasal i.d. inoculation of TGE virus.

The data (table 8) indicate clearly that the anti-TGE serum had no protective effect when given orally. Both pigs died within four days of inoculation. Essentially the same results were obtained with the virus control pigs which died on the fourth and fifth days following inoculation. A definite protective effect was obtained from the serum injected intraperitoneally. Although both pigs had typical signs of TGE both recovered. Two weeks after diarrhea ceased, these pigs appeared in excellent health. Demonstrable TGE virus was not present in the feces of these pigs one and two weeks after diarrhea had ceased.

DISCUSSION

The studies conducted in this laboratory on TGE in disease-free antibody-devoid swine indicated that the causative agent was a filterable virus with an affinity for epithelial tissues. Multiplication of the

TABLE 3—Quantitative Studies of Transmissible Gastroenteritis (TGE) Infection in 2-Hour-Old Baby Pigs

Time from inoculation* until killed (hr.)	Titer (in negative logs)** of TGE virus in tissues listed											
	Nasal mucosa	Lung	Cardiac stomach	Pyloric stomach	Duodenum	Jejunum	Ileocecal pouch	Kidney	Liver	Testis	Spleen	Blood
4	0	0	0	0	—1	0	0	0	0	0	0	0
8	0	—4	—1	0	—3	—2	0	0	0	0	0	0
12	0	—4	0	0	—3	—2	0	0	0	0	0	0
14	0	—5	0	0	—4	—2	0	0	0	0	0	0
16	—1	—5	0	0	—2	—2	0	0	0	0	0	0

* Used approximately 10 minimum infectious doses of TGE virus for 5-day-old pigs.

** Assay made by intranasal inoculation of 5-day-old pigs with tenfold dilution of tissue suspensions. Presented as negative exponent. Would ordinarily read 10^{-2}, 10^{-3}, 10^{-4}, etc.

0—Less than 1 infectious dose of TGE virus in 10^{-1} dilution of tissue suspension.

TABLE 4—Quantitative Studies of Transmissible Gastroenteritis (TGE) Infection in 6-Day-Old Baby Pigs

Time from inoculation* until killed (hr.)	Titer (in negative logs)** of TGE virus in tissues listed											
	Nasal mucosa	Lung	Cardiac stomach	Pyloric stomach	Duodenum	Jejunum	Ileocecal pouch	Kidney	Liver	Testis	Spleen	Blood
12	—4	0	—2	—2	—3	—4	—1	0	0	0	0	0
24	—5	0	—2	—2	—7	—6	—4	—1	0	0	0	0
48	—3	0	—3	—4	—5	—5	—3	—2	—1	0	—3	—1
72	—3	—1	—2	—4	—5	—5	—3	0	—1	0	NT	—1

* Used approximately 100 infectious doses of TGE virus for 5-day-old pigs; ** assay made by intranasal inoculation of 5-day-old pigs with tenfold dilution of tissue suspensions. Presented as negative exponent. Would ordinarily read 10^{-3}, 10^{-3}, 10^{-4}, etc.; NT—not tested.

agent in nasal mucosa and lung epithelium had not been previously reported.

These studies also have shown that TGE behaves quite differently in pigs of different ages. Transmissible gastroenteritis in newborn pigs and pigs up to a week old caused mortalities at or near 100 per cent. A maturation resistance developed so that, under the conditions of this study, 4-week-old pigs underwent inapparent TGE infections. Previous encounter with the causative agent was not involved in maturation resistance.

A part of maturation resistance in TGE was due to changes in the tissues themselves. The findings with lung tissues illustrated this very well. The lung tissues of newborn pigs were readily attacked by TGE virus. A single intranasal i.d. of virus was sufficient to initiate infection with rapid multiplication of the virus in lung tissue. By the time pigs were 5 days old, lung tissue was refractory to 10^4 i.d. of TGE virus.

Further evidence of local tissue change contributing to maturation resistance was the absence of symptoms in 24-day-old pigs, although infection occurred as demonstrated by viremia in 4 of 4 pigs. The intestinal epithelia were refractory to the agent or physiologically capable of handling the virus without sufficient increase in intestinal fluids to result in diarrhea.

The finding that virus multiplied in the lung of newborn swine and in the nasal mucosa of older swine pointed to spread of TGE by means of contaminated air. Lee, Moro, and Baker [2] reported inability to house normal pigs and pigs infected with TGE in the same room even though great care was taken to prevent tracking of the agent from one pig to the other. This finding had also been informally reported by other workers and was the motivating force in 1950 to begin housing our swine in individual isolation units [4] for disease studies. With either the lung or nasal mucosa of pigs less than a week old vulnerable to attack by TGE virus, any airborne virus obviously has a potential site for multiplication and thus a means of continuing itself. This, of course, was in addition to

TABLE 5—Relationship of Age of Host to Susceptibility and Manifestation of Signs on Inoculation with Varied Doses of Transmissible Gastroenteritis (TGE) Virus

Age of host* (days)	Dilution of standard TGE suspension	Observation for signs of vomition (V) or diarrhea (D) at time indicated (hours)								
		24	36	48	72	96	120	148	176	200
11	10^{-2}	0	VD	VD	D	D	D	D	D	D
	10^{-3}	V	V	VD	D	D	D	D	D	D
	10^{-3}	V	VD	VD	VD	Dead				
	10^{-4}	0	VD	VD	D	D	D	D	D	D
17	10^{-2}	0	0	D	D	D	D	D	D	D
	10^{-3}	V	V	V	VD	D	D	D	D	D
	10^{-3}	0	0	V	D	D	0	0	0	0
	10^{-4}	V	VD	VD	D	D	D	D	D	D
24	10^{-2}	VB+	0	OB+	OB+	OB+	0	0	0	0
	10^{-3}	0	0	I	OB+	OB+	0	0	0	0
	10^{-3}	0	0	I	0	0	OB+	OB+	0	0
	10^{-4}	0	0	I	OB+	OB+	OB+	0	0	0

* Disease-free antibody-devoid pigs reared in isolation units; 0—no signs of illness; B+—blood contained TGE virus as shown by intranasal inoculation of 5-day-old pigs, but hosts showed no signs of TGE. Blood samples taken from anterior vena cava of all pigs at each interval except 36 hr. All tested for TGE; I—pigs showed inappetence by failing to consume usual amount of milk.

TABLE 6—Demonstration of Serum Neutralizing (SN) Antibody to Transmissible Gastroenteritis (TGE) Virus

Age at inoculation (weeks)	No. of pigs	No. of exposures to virus	Harvest after last exposure (days)	Type of serum	Number of serums SN−	Number of serums SN+
2	3	1	15	Preinoculation	3	0
				Postinoculation	3	0
16	2	3	21	Preinoculation	2	0
				Postinoculation	0	2

SN− = Serum did not contain sufficient antibody to neutralize 1,000 intranasal i.d. of TGE virus; TGE infection occurred in test pigs.
SN+ = TGE virus neutralized; test pigs remained normal.

virus-contaminated fecal materials as a source of virus.

From the standpoint of control of TGE by chemotherapeutic agents or serums, the very nature of the disease makes it difficult to handle. Onset is so rapid that treatment would need to begin at birth even with an effective chemotherapeutic agent! Thus far no chemotherapeutic agent which is effective against TGE is available.

A potential decrease in the intensity of TGE is possible through absorption of TGE antibody-rich colostrum from the dam or by administration of immune TGE antiserum. Colostral lactoglobulins may result through naturally occurring TGE and recovery of the dam, or be induced by artificially infecting the dam several weeks before farrowing.[6,7] One difficulty, however, is that in an environment within which the TGE virus is present, it is almost impossible to obtain enough antibody to entirely prevent infection. Circulating antibody does not necessarily reach the exposed outer surfaces in sufficient concentration to prevent infection.[8] It may protect the host by modifying the epithelial infection and by preventing damage to vital tissues by neutralizing invading virus to prevent or minimize viremia.

Transmissible gastroenteritis obtained its name from the typical field disease among young swine. Under field conditions, the gastrointestinal tract may become severely inflamed. Under laboratory conditions with disease-free antibody-devoid pigs, however, TGE virus caused severe vomiting and diarrhea with death in pigs less than a week old without gross evidence of inflammation in the gastrointestinal tract. This observation, plus inapparent experimental infections in 24-day-old pigs, was contradictory

TABLE 7—Serum Neutralizing Capacity of Specific Transmissible Gastroenteritis (TGE) Antiserum

	Mixture of 1,000 infectious intranasal doses of TGE virus and specific TGE antiserum diluted:					
Trial	1:4	1:8	1:16	1:32	1:64	1:128
First	—	—	—	—	NT	NT
Second	NT	NT	—	—	TGE*	TGE

— Serum neutralized virus, test pig remained normal; * serum did not neutralize virus; NT = not tested.

TABLE 8—Demonstration of Protection against Transmissible Gastroenteritis (TGE) Virus from Specific Anti-TGE Swine Serum

Days following challenge with TGE virus*	Normal litter mate controls		Anti-TGE serum given as follows: 50 ml. orally 4 hr. after birth		20 ml. intraperitoneally 24 hr. after birth**		Virus control	
1	0	0	0	D	0	D	0	D
2	0	0	D	VD	V	VD	VD	VD
3	0	0	VD	VD	D	D	VD	D
4	0	0	Dead	Dead	D	D	Dead	D
5	0	0	....	....	D	D	....	Dead
6	0	0	....	....	D	D	....	....
7	0	0	....	....	VD	D	....	....
8	0	0	....	....	VD	D	....	....
9	0	0	....	....	VD	D	....	....
10	0	0	....	....	D	D	....	....
11	0	0	....	....	D	D	....	....
12	0	0	....	....	0	0	Both pigs recovered.	
13	0	0	....	....	0	0	Killed at 28 days;	
14	0	0	....	....	0	0	tissues grossly normal	

* Normal controls excepted, pigs given 1,000 intranasal i.d. of TGE virus twenty-four hours after birth; ** 10 ml. at 20 hours and 10 ml. at 24 hours; 0—no signs of illness; V—vomition; D—diarrhea.

to reports of relatively severe TGE with diarrhea in older animals in the field. Two obvious possibilities are (1) our TGE virus was of relatively low virulence, and (2) secondary organisms such as bacteria, other viruses, or phage may have aided TGE virus in producing more severe diseases. The second consideration seems most feasible as Reber and Whitehair [9] produced vomition and diarrhea in 26-day-old pigs with the same strain of TGE with which we caused only inapparent TGE in 24-day-old disease-free antibody-devoid pigs. Their pigs had nursed the dam two days before being placed in a separate environment preceding inoculation with the virus. Thus, there was an opportunity for influence of TGE infection by secondary organisms common to the dam's environment.

The data presented in this paper demonstrating antibody by both serum neutralization and host protection tests confirm previous reports [5, 7] that specific antibody does develop following TGE infection. The inability of Lee *et al.* to demonstrate TGE antibody was in all probability due to the use of serums from immature pigs. Pigs less than 4 weeks old are poor antibody producers.

The administration of 50 ml. of specific anti-TGE serum orally to pigs failed to give them any protection to TGE whereas lesser amounts given intraperitoneally gave some protection. It is probable that serum given orally four hours after birth was not absorbed and was thus not useful to the pigs against TGE infection. Antibody absorption in pigs has been shown to occur early with maximal titers present within three to six hours after birth.[10] Even colostral antibody absorption may be blocked within a short time after birth, which would be a possible explanation for failure to get protection by oral administration of anti-TGE serum. Another explanation might be the inability of the newborn pig at any time to absorb serum globulins in contrast to absorption of lactoglobulins from colostrum.

Summary

Some characteristics of transmissible gastroenteritis (TGE) of swine were determined by study of the disease in young disease-free antibody-devoid pigs. The findings are summarized as follows.

The infective TGE agent passed a 230 mμ gradacol membrane but not a 170 mμ membrane. Its particle size was estimated at 200 mμ.

Five-day-old pigs were refractory to large doses of the TGE agent on intratracheal instillation and intraperitoneal injection. They were readily susceptible to the virus on intranasal and intragastric instillation.

Although the TGE agent did not ordinarily multiply in lung tissue of 6-day-old pigs following intranasal instillation, it multiplied readily in the lung of pigs inoculated when 2 hours old. The opposite occurred in the nasal mucosa. Whereas the virus readily multiplied in the nasal mucosa of 6-day-old pigs, it did not in 2-hour-old pigs. The agent multiplied readily in all portions of the gastrointestinal tract. Viremia forty-eight or more hours after inoculation was common. A maturation resistance to TGE occurred in pigs with advancing age. Mortality approached 100 per cent in pigs less than a week old. Obvious infections occurred in 11- and 17-day-old pigs but the mortality was low. In 24-day-old pigs, TGE infection occurred but was inapparent.

Specific anti-TGE serum was produced in 16-week-old pigs. It contained sufficient antibody to neutralize TGE virus *in vitro* and to protect the life of pigs *in vivo*.

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Toxoplasmosis. VI. Isolation of Toxoplasma from Milk, Placentas, and Newborn Pigs of Asymptomatic Carrier Sows

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WHEN TOXOPLASMOSIS in swine was discovered on an Ohio farm in 1951, losses were greatest among the young pigs.[3] Congenital infection was known to occur in human babies, puppies, and mice so the possibility of intrauterine transmission of porcine toxoplasmosis was investigated. This report concerns the study of pregnant sows in the herd infected with Toxoplasma as well as pregnant gilts experimentally infected with *Toxoplasma gondii*.

LITERATURE REVIEW

The only proved natural mode of transmission of toxoplasmosis in man is the intrauterine transfer of the organism from mother to fetus. Mothers of such infected babies apparently suffer no ill effects from the presence of Toxoplasma in their tissues but the protozoon causes serious or fatal illness in the child.[6] A similar congenital transmission as well as milk-borne transfer of the disease has been demonstrated in mice.[2] Intrauterine transmission of canine toxoplasmosis was demonstrated by isolation of the protozoon from 23 of 28 pups born of 5 bitches experimentally infected at different stages of pregnancy.[1] Isolation of organisms from placenta and from allantoic fluid provided further evidence of *in utero* transmission. The protozoon was excreted in the milk of bitches [1] and of a cow.[5]

MATERIALS AND METHODS

Seven sows were used in this study. Three were 2-year-old pregnant Hampshires which weighed about 500 lb. They were in a herd where toxoplasmosis was diagnosed in pigs.[3] Four 250-lb. gilts were purchased locally; 3 were pregnant. The fourth gilt had 8 pigs 6 days of age and served as an uninoculated control. On the basis of physical examinations, daily temperature recordings, and hematological studies all sows and gilts were healthy. They were housed in separate pens and fed a balanced commercial hog feed.

Serological studies were conducted according to the method of Sabin and Feldman who described dyes as microchemical indicators of a new immunity phenomenon affecting Toxoplasma.[4] Toxoplasma of canine origin as well as from swine were used as the antigen. Toxoplasmin used for intradermal tests was prepared from a cell-free filtrate of *T. gondii* isolated from a dog.

The inoculum consisted of fresh pig tissues known to be infected with Toxoplasma organisms of porcine origin.[3] Liver, spleen, kidney, heart, and brain from infected pigs were prepared in a Waring blendor. The final inoculum consisted of a 1:10 tissue suspension in saline.

During parturition the udder of each sow was scrubbed with soap and water, then washed with alcohol to permit aseptic collection of colostrum. No pigs were allowed to nurse until after colostrum was obtained. At the same time, representative placentas and pigs from each litter were collected aseptically from the vagina. These newborn pigs were immediately destroyed; liver, spleen, kidney, lung, heart, and brain tissues were aseptically collected for mouse passage attempts to isolate Toxoplasma. When the sows were destroyed for necropsy, liver, spleen, kidney, heart, lung, brain, and skeletal muscle specimens were aseptically collected for mouse inoculation.

White Swiss mice, 8 weeks or older of both sexes, were used for isolation of Toxoplasma. Small pieces of tissue to be tested for Toxoplasma were finely ground in a dry, sterile mortar. Sterile saline was added to make a 1:10 suspension and 1 ml. was injected intraperitoneally into mice.

For intracerebral inoculation, the mice were anesthetized and 0.02 ml. of the suspension was deposited in the subdural space through a 26-gauge needle.

Two mice were inoculated with each organ and 8 mice with milk and other body fluids. Four days postinoculation, they were checked for Toxoplasma by abdominal paracentesis. When fluid was present, a Wright-stained smear was examined for Toxoplasma. This procedure was repeated two or three times weekly. Failure to obtain Toxoplasma in the first group of mice was followed by three blind passages of inoculum made of brain, heart, lung, and liver of mice before being declared negative.

For micropathology, thin sections of organs from each body system, except the skeletal, were fixed in 10 per cent formol-saline solution, embedded in paraffin, sectioned at 6 μ and stained by hematoxylin and eosin, Masson's trichrome, van Gieson's, and von Kossa's techniques.

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Results

Sows from Farm Where Toxoplasmosis Enzootic Occurred.—Sow 467 was healthy and nonpregnant at the time blood was obtained and the toxoplasmin intradermal test was applied on Jan. 12, 1952. One-tenth milliliter of toxoplasmin was injected intradermally into the upper eyelid and at the base of the ear. When the sites of the test were examined forty-eight hours later, edematous swellings measuring 10 mm. in diameter were found on both. When serum was tested for Toxoplasma antibodies a titer of 1:10 was obtained. This sow was bred on Jan. 13, 1952, by a boar which was insensitive to toxoplasmin although he was in the herd during the 1951 outbreak [3] of toxoplasmosis. On Jan. 31, 1952, the sow was removed from the farm and isolated in a research building in which animals had never been housed. During gestation, she remained asymptomatic; the hemogram did not deviate from the normal range and 3 pigs, all living, were farrowed on May 7, 1952. Two of the pigs and the placenta were collected aseptically from the birth canal for mouse passage tests. Toxoplasma organisms were recovered from the organs of both pigs but not from the placenta. Toxoplasma were also isolated from colostrum collected at the time of parturition before the sow was nursed. The surviving pig nursed its mother for two weeks after which the sow was destroyed for necropsy and the pig was transferred to another lactating sow. Although this pig was from an infected dam and nursed her during the time her colostrum contained Toxoplasma, the pig remained asymptomatic and gained normally during a five-months' observation.

Throughout gestation and the two weeks of lactation prior to euthanasia, the dam (467) remained asymptomatic. No lesions were seen at necropsy; liver, spleen, lung, ovary, heart, and brain were collected and individually tested for viable Toxoplasma by mouse passage. Of the organs tested, Toxoplasma were isolated only from the heart. Micropathological examination of the thoracic and abdominal organs and of the nervous system of the sow and 2 of her pigs revealed no significant lesions (table 1).

Sow 395 was bred on Dec. 23, 1951, and when tested on Jan. 12, 1952, her blood serum was positive for Toxoplasma antibodies at a titer of 1:10. Toxoplasmin injected intradermally at the lip of the vulva and the edge of the upper eyelid resulted in reactions at each site measuring 8 mm. in diameter (fig. 1). This sow was removed from the farm and placed in isolation. She was asymptomatic during gestation and farrowed 1 dead and 9 living pigs on April 8, 1952. The placenta, 1 dead pig, and 3 live pigs were collected directly from the vagina for mouse passage tests. Toxoplasma were isolated from the kidney, brain, and heart from each of the 3 live pigs but not from the dead pig or the placenta. Colostrum collected aseptically before the sow was nursed also contained Toxoplasma organisms.

The 6 surviving pigs nursed their mother for thirty-two days after which the sow was destroyed for necropsy. When the pigs were 25 days of age, their serums were tested and 2 of the 6 pigs were found to

TABLE 1—Recovery of Toxoplasma from Naturally and Experimentally Infected Sows and Their Pigs

Sow (No.)	Day of gestation when inoculated	Inoculum* (ml.) and route	No. of pigs farrowed live/dead	Toxoplasma isolated: No. pigs examined/ No. infected	Placenta	Milk	Organs of sow
467	Natural infection		3/0	2/2	—	+	+
395	Natural infection		9/1	4/3	—	+	—
369	Noninfected sow from farm		12/0	1/0	—	**	—
396	107	IM—80 SQ—80 oral—80	8/0	3/0	—	+	—
335	84	SQ—120 oral—120	5/2	3/0	+	—	—
397	108	IM—120 SQ—120	6/0	4/0	+	—	—
336	Uninoculated control		8/0	8/0	**	**	—

* Inoculum consisted of liver, spleen, heart, kidney, and brain from pigs infected with Toxoplasma. IM—intramuscular; SQ—subcutaneous; ** not examined.

have Toxoplasma antibodies. Although organisms were isolated from tissues of 3 litter mates and Toxoplasma were present in the milk, all 6 surviving pigs showed no

Fig. 1—Sow with naturally occurring toxoplasmosis showing intradermal toxoplasmin reaction on the left lip of the vulva (arrow). The reaction consisted of edema measuring 8 mm. in diameter thirty-six hours after intradermal injection of 0.1 ml. of toxoplasmin.

illness. Organs from the sow revealed no gross or microscopic lesions and no organisms were recovered by the mouse technique from the brain, heart, lung, spleen, kidney, and ovary. Micropathological study of tissues from 2 pigs killed at the time of birth revealed focal necrosis in the liver and groups of microglia in the cerebrum. The focal accumulations of glial cells were not unlike those frequently seen in the brains of healthy pigs; however, the liver necrosis may have been caused by Toxoplasma.

Sow 369 was insensitive to toxoplasmin and no Toxoplasma antibodies were detected in her serum collected on Jan. 12, 1952. She was bred in December, 1951, and isolated from the herd on Jan. 31, 1952. She remained asymptomatic until euthanasia on April 10, 1952. At necropsy, 1 runt and 11 well-developed pigs were removed from the uterus and tissues were collected. Toxoplasma were not recovered from the sow, 1 of her pigs or the placenta. No gross or microscopic lesions were found in the sow or the pigs.

Pregnant Gilts Inoculated with Toxoplasma Gondii.—During preinoculation examinations, 3 pregnant gilts (table 1) were found to be in good body condition, healthy, and active. Their serums contained no Toxoplasma antibodies and all were insensitive to toxoplasmin. Separate pens were provided for each gilt.

Gilt 335 was given 120 ml., subcutaneously, and 120 ml., orally, of a 1:10 suspension of pig tissues infected with Toxoplasma. The inoculation was made on the eighty-fourth day of gestation and six days later the body temperature rose to 104 F. The fever subsided in twenty-four hours and no other signs appeared during gestation. Five mature, living pigs and 2 small mummified pigs were farrowed at the expected time. Colostrum, placenta, and 3 pigs destroyed at the time of birth were used for mouse passage. *Toxoplasma gondii* was recovered from the placenta; however, no organisms were isolated from the colostrum or organs of the pigs. No lesions were seen in the pigs. Thirty-eight days after parturition the dam (335) was destroyed for laboratory study. No lesions were found and no organisms were recovered from her organs.

Gilt 396 received 80 ml. of infected tissue suspension by each of three routes, intramuscular, subcutaneous, and oral. The inoculation was made on the 107th day of gestation and signs of illness appeared after an incubation period of four days. She became depressed, listless, and developed a mucopurulent nasal discharge which persisted until the time of parturition.

Eight living pigs were born at full term. Colostrum, placenta, and organs from 3 pigs killed at the time of birth were used in mouse passage experiments. Toxoplasma organisms were recovered from the colostrum but not from the placenta or pigs. When the gilt was destroyed seven weeks after farrowing, the organ systems were free of lesions and no Toxoplasma were found.

Gilt 397 was given 120 ml. of infected suspension by both the intramuscular and subcutaneous routes on the 108th day of pregnancy. No illness was observed and 6 living pigs were farrowed six days later. Three pigs were destroyed at birth and 1 pig was killed when 6 weeks of age. Toxoplasma was recovered from the placenta; no organisms were isolated from the milk or from organs of the sow and pigs.

Gilt 336, an uninoculated control, was asymptomatic throughout the observation period of seven weeks. During this interval she remained insensitive to toxoplasmin

on three tests and no Toxoplasma antibodies were detected in two serum samples. Neither lesions nor Toxoplasma were found in her organs.

Discussion

The invasiveness of *T. gondii* was demonstrated by the recovery of the organism from the placenta and the milk of gilts inoculated subcutaneously, intramuscularly, and orally. The fact that pregnant gilts were inoculated at the terminal stages of pregnancy may explain the absence of infection in their pigs. Previous studies of bitches at this laboratory [1] showed that infection midway in the gestation period resulted in fatal infection while inoculation late in pregnancy resulted in less serious disease. Further studies of sows are required to determine the effects of infection during the early weeks of pregnancy.

Serum of 1 sow from the infected herd contained no antibodies and her tissues as well as her pigs were free of infection. Serum of 2 sows from this herd possessed antibody titers of 1:10 and subsequently both were found to be carrying Toxoplasma; thus, the serological test may prove to be a practical means for detection of carrier animals. The source of human infection with Toxoplasma has not been established. In this study, *T. gondii* were isolated from edible parts of the carcass of healthy swine. Therefore, swine may eventually be implicated in the epidemiology of toxoplasmosis in man in a manner similar to the trichinosis cycle.

Summary

Toxoplasma gondii was isolated from 2 pigs collected aseptically as they emerged from the birth canal, as well as from the milk and the heart of a naturally infected sow which was consistently asymptomatic. Toxoplasma organisms were isolated from the milk and from 3 of 4 pigs from a second apparently healthy, naturally infected sow. Thus pregnant, apparently healthy, sows carrying Toxoplasma transmitted the infection to their pigs *in utero*. Furthermore, the protozoon was carried in edible portions of the carcass.

Toxoplasma was isolated from the milk at the time of parturition of 2 naturally infected sows and from the milk of 1 experimentally infected gilt. The organism was present in the placentas from 2 of 3 experimentally infected gilts.

Inoculation of gilts late in gestation (84th to 108th day) did not result in congenital infection in their pigs.

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Studies on Infectious Atrophic Rhinitis. IV. Characterization of a Pleuropneumonia-like Organism Isolated from the Nasal Cavities of Swine

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IN THE COURSE of work on infectious atrophic rhinitis in swine a pleuropneumonia-like organism (PPLO) was isolated. Previous reports of this work [6-9] have been published. Additional studies on this agent allow more complete characterization.

METHOD OF PROCEDURE

Bacteria-free filtrates of specimens of nasal turbinate from swine were prepared by the following technique. The turbinates were removed from the nasal cavity and ground in a mortar with alundum. Enough tryptose broth was added to make a 1 to 2 per cent tissue suspension. The suspension was centrifuged at 2,000 r.p.m. for fifteen minutes and the supernatant fluid clarified by filtration through a Selas No. 10 filter. This material was then filtered through a Selas No. 02 filter at a negative pressure of 8 to 10 inches of mercury. A Selas No. 02 filter has proved to be effective in preparing filtrates containing the swine PPLO free from bacteria. Carter and McKay [2] verified the existence of a PPLO in the nasal cavities of swine and reported it could be grown in thioglycolate medium enriched with horse serum. In a few trials using this medium, it was not possible to obtain satisfactory growth so a chicken infusion-chicken serum medium developed by Hofstad [3] for the cultivation of the PPLO associated with chronic respiratory disease of poultry was tried. It was possible to obtain growth of the swine PPLO in this medium and to evolve a medium that was considered to give more satisfactory growth. The latter medium is prepared by passing fresh ox hearts with the fat trimmed off through a meat grinder and allowing 1 part ground heart to infuse in 2 parts distilled water for twelve hours at approximately 5 C. The infusion is heated in a boiling water bath for thirty minutes and filtered through coarse filter paper. The infusion is adjusted to pH 7.8 with 0.10 normal sodium hydroxide (NaOH) after which 0.2 per cent bacto hemoglobin and 0.5 per cent of sodium chloride (NaCl) are added. The infusion is heated a second time in a boiling water bath for thirty minutes. When the medium has cooled, it is filtered through filter paper and a Seitz clarifying pad. Then 20 per cent heat-inactivated (56 C. for 30 minutes) chicken serum is added and the complete medium sterilized by filtration through a Selas No. 03 filter. It is dispensed into sterile 13 by 125 mm. screw-top tubes in 7- to 8-ml. amounts. The sterility is checked by incubation of the tubed medium at 37 C. for twenty-four hours. The tubed medium is stored at 5 C. until needed.

The swine PPLO was also isolated from contaminated material by the addition of enough thallous acetate to make a final concentration of 1:4000 and the addition of 2,000 to 3,000 units of crystalline penicillin per milliliter of medium.

RESULTS

A slight turbidity indicating growth of the swine PPLO usually occurs in the ox heart infusion-chicken serum medium in twenty-four to forty-eight hours. Uninoculated control tubes should be included in each transfer for comparison. It is desirable to transfer this organism at 48- to 72-hour intervals. The usual amount of inoculum transferred each time was 1 ml. In some 24-hour-old cultures, growth was present only in the upper portion of the medium but by forty-eight hours the turbidity was usually uniform throughout the medium. Cultures transferred after seven days were usually nonviable. No pellicle formation was observed in any of the isolates of this organism, although sometimes there was a small amount of sediment present. Growth of the PPLO was verified by examination of smears prepared from the sediment present in the cultures after centrifugation at 2,500 r.p.m. for fifteen minutes. These smears were stained for one and a quarter hours with Giemsa's stain.

The swine PPLO can be grown in embryonating chicken eggs by either the yolk sac or chorioallantoic sac route of inoculation. Since only about one half of the inoculated chicken embryos develop lesions or die, at least 6 embryos should be used for each inoculum. When 7- to 8-day-old chicken embryos are inoculated, there is usually no mortality due to this organism during the

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first four days. Embryo mortality begins on the fifth day and reaches the peak on the sixth or seventh day and may continue for as long as thirteen days postinoculation. The lesions observed in the chicken embryos correlate with the age of the embryo at the time of death. Embryos inoculated at 10 days of age and dead four days postinoculation may have the same lesions as embryos inoculated at 7 days of age and dead seven days postinoculation but the older embryos are less susceptible to this agent. Chicken embryos inoculated at 7- to 8-days of age and dead on the fourth to sixth day postinoculation usually have diffuse hyperemia of the skin, the liver, and kidneys. Some of the chicken embryos dead the sixth day postinoculation have mild myocarditis but this lesion is more common in those dead on the seventh day postinoculation. The embryos dead the seventh day postinoculation usually show mild hyperemia. Those dead on the eighth to the thirteenth day postinoculation often have severe pericarditis and small necrotic foci in the liver. When smears prepared from the pericardial exudate are stained with Giemsa's stain and examined, numerous small coccobacillary objects typical of PPLO are consistently observed (fig. 1 and 2). All ages of chicken embryos succumbing to this organism have kidney hyperemia. Other lesions occasionally observed in the dead chicken embryos are peritonitis, ulceration of the skin in the occipital region, and edema. In only 1 of several thousand chicken embroys inoculated with this agent has a distinct joint abscess been observed. Amnioallantoic fluid from inoculated chicken embryos with no gross lesions when necropsied ten days postinoculation has produced typical lesions when inoculated into normal 7-day-old chicken embryos. Therefore the routine procedure has been to make a second chicken embryo passage of amnioallantoic fluid before considering a specimen negative for the swine PPLO.

Titration trials conducted in chicken embryos with this organism do not have a distinct end point. The end point usually extends through three tenfold dilutions. It was found that the infective dose ($i.d._{50}$) for chicken embryos of a chorioallantoic membrane pool from infected chicken embryos was $10^{-6.5}$, of yolk sac membrane $10^{-4.6}$, of embryo proper $10^{-4.8}$, and of amnioallantoic fluid $10^{-5.2}$. No detectable acid or gas formation occurs when 1 per cent lactose, sucrose, dextrose, maltose, or mannite plus 0.01 per cent phenol red is added to the fresh ox heart infusion-chicken serum medium. Since PPLO do not always produce detectable pH changes the technique of Somerson and Morton [5] and Lecce and Morton [4] of using triphenoltetrazolium chloride to act as an indicator of electron transfer has been employed. It was observed that more electron transfer occurred in the base medium than in the base medium containing the previous enrichments

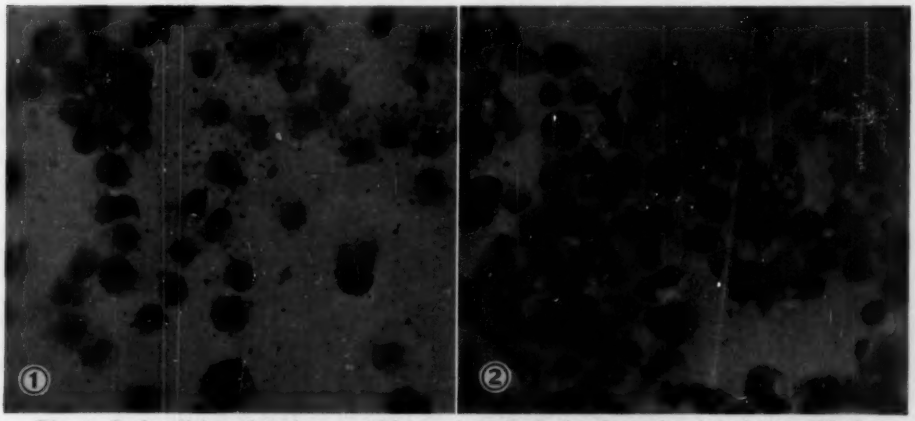

Fig. 1—Pericardial exudate from a chicken embryo dead nine days after inoculation with the swine pleuropneumonia-like organism. Giemsa's stain. x 980.
Fig. 2—Pericardial exudate from a chicken embryo dead nine days after inoculation with the swine pleuropneumonia-like organism. x 980.

and 0.5 per cent ethyl alcohol, suggesting that the enrichments used actually had a slight inhibitory effect on growth of this organism.

The swine PPLO can be grown on ox heart infusion-chicken serum medium to which 1.5 per cent agar has been added. The colonies are very minute. At forty-eight hours, they impart a velvetlike appearance to the surface of the medium and are readily observed under a magnification of 20. The diameter of the colony is 0.01 mm. to 0.1 mm. Not all of the colonies develop at a uniform rate. A week after inoculation, a few colonies are still developing. This may cause confusion when attempting to determine the presence of more than one PPLO in a specimen. The colonies of the swine PPLO are small and glistening with regular margins. They have little tendency to coalesce. There is an indefinite central elevation that becomes granular as the colony ages. The colonies remain distinct and have no tendency to spread or to form a film (fig. 3). Examination of smears of this organism stained with Giemsa's stain reveals the predominant form to be minute coccoid rods 0.3 μ to 0.6 μ in length and 0.3 μ or less in width. Rod forms up to 1.0 μ in length and some small dense spherical forms about 0.2 μ in diameter are observed. In addition, ring forms 0.3 μ to 0.6 μ in diameter with a definite decrease in the density of the central portion or a vacuolated central portion are observed (fig. 4).

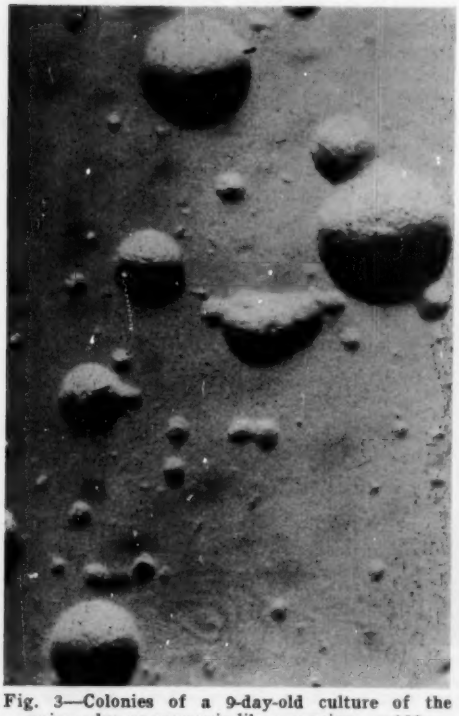

Fig. 3—Colonies of a 9-day-old culture of the swine pleuropneumonia-like organism. x 120.

Examination of cultures of the swine PPLO with the aid of the electron microscope reveals the organisms are irregular spheres, many of which have central vacuoles. The organism has little rigidity of the cell wall as evidenced by the degree of flattening (fig. 4 and 5). No filamentous forms are observed. This organism is not acid fast. It stains faintly gram-negative with Gram's stain and is stained blue with Macchiavello's stain.

The swine PPLO withstands 56 C. for thirty minutes but not for sixty minutes. It withstands storage at —40 C. for more than ten months. Cultures of this organism are usually nonviable when stored at 4 C. for three weeks. The addition of 50 per cent glycerin to the cultures stored at 4 C. appears to have little influence on their survival. Cultures of this organism lyophilized and stored at 4 C. retain their viability for more than a year.

An attempt was made to elicit neutralizing antibodies against the swine PPLO in rabbits, chickens, and pigs but no significant neutralizing antibody titer could be demonstrated in any of these species.

The lesions produced by the swine PPLO in chicken embryos resemble those produced by the PPLO associated with chronic respiratory diseases of poultry. Therefore, determination of the pathogenicity of the swine organism for poultry was desirable. Chickens 3 to 8 weeks of age and turkey poults 6 to 8 weeks of age were inoculated intranasally, intratracheally, by way of the intraorbital sinus, and by way of the conjunctival sac with chicken embryo amnio-allantoic fluid containing the swine PPLO. These inoculated birds did not develop respiratory symptoms during a three-week observation period and had no gross lesions at the end of this period.

The swine PPLO failed to produce lesions or symptoms in 6- to 8-week-old mice inoculated intraperitoneally, intracranial-

ly, intravenously, and by inhalation while under ether anesthesia. These mice were observed for eleven days postinoculation. Guinea pigs inoculated intraperitoneally with this organism were normal when necropsied one week postinoculation. Rabbits were likewise normal when necropsied one to seven weeks after being inoculated intravenously with this organism. A ten-week-old calf was inoculated intraperitoneally with material containing this agent. No temperature elevation occurred

TABLE 1—Occurrence of the Swine Pleuropneumonia-like Organisms (PPLO) in the Nasal Cavities of Normal Swine and of Those Affected with Infectious Atrophic Rhinitis

	Turbinates positive for PPLO		Turbinates negative for PPLO		
	(No.)	(%)	(No.)	(%)	Total
Swine from field cases					
Atrophic turbinates	18	69.2	8	30.8	26
Normal turbinates	18	66.7	9	33.3	27
Swine from V.M.R.I.* herds					
Atrophic turbinates	46	65.7	24	34.3	70
Normal turbinates	22	47.8	24	53.3	46
Swine inoculated intranasally with PPLO					
Atrophic turbinates	0	.....	0	.....	0
Normal turbinates	12	70.6	5	29.4	17

* Veterinary Medical Research Institute.

and no significant lesions were present when the calf was necropsied two weeks postinoculation. A 5-month-old lamb was inoculated intraperitoneally with 5 ml. of a 48-hour ox heart infusion-chicken serum culture of the swine PPLO and developed no temperature elevation. No lesions were present when this lamb was necropsied one week postinoculation.

As has been reported previously,[1,7] the intranasal inoculation of baby pigs with the swine PPLO frequently resulted in the organism becoming established in the nasal cavity but did not produce gross atrophy of the nasal turbinates. Fibrinous peritonitis, pleuritis, pericarditis, and arthritis frequently occur in pigs inoculated intraperitoneally with this organism. Similar lesions in field cases have been shown to be due to this organism. In working with the swine PPLO in pigs, it was found that when pigs over 6 weeks of age were inoculated intraperitoneally relatively mild lesions occurred, whereas pigs under 6 weeks of age usually developed severe visceral lesions. The most severe lesions occurred

Fig. 4—The swine pleuropneumonia-like organism shadow cast with gold. x 5,600.

in pigs that had a concurrent anemia. The organism has also been recovered from pneumonic swine lungs but its role is difficult to evaluate since a considerable number of animals with lungs containing this organism also had the organism present in the nasal cavity.

The initial isolations of the swine PPLO were made from atrophic turbinates. As additional specimens were examined it was found that this organism also occurred in normal appearing turbinates. A total of 96 swine specimens with gross turbinate atrophy were examined for the presence of the swine PPLO with 64 (66.7%) being positive and 32 negative. Of 90 grossly

Fig. 5—The swine pleuropneumonia-like organism shadow cast with gold. x 30,000.

normal swine nasal cavities examined, 52 (67.8%) were positive for the swine PPLO while 38 were negative. A summary of these findings is presented in table 1.

It is proposed that this PPLO of swine be named *Mycoplasma hyorhinis* (n. sp.). The use of generic name Mycoplasma is based on the discussion contained in the International Bulletin of Bacteriological Nomenclature and Taxonomy (1955).[10] The species name *hyorhinis* indicates that this organism is common in swine nasal cavities.

SUMMARY

The natural habitat of the swine pleuropneumonia-like organism (PPLO) appears to be the nasal cavities of swine. It is readily filterable through a Selas No. 02 filter and can be grown in serum enriched fluid and solid medium. It produces colonies 0.01 mm. to 0.1 mm. in diameter with an entire periphery and a smooth, glistening appearance. Some of the colonies have a small central elevation. The central portion of the colony becomes granular as the colony ages. The individual organisms appear as minute coccoid rods 0.3 to 0.6 μ in size and are frequently vacuolated. This organism is stained well by Giemsa's stain but poorly by Gram's stain. Macchiavello's stain imparts a distinct blue color to the organism. It is relatively insensitive to penicillin or 1:4000 concentration of thallous acetate. This organism produces no detectable acid or gas from lactose, sucrose, dextrose, mannite, maltose or 0.5 per cent ethyl alcohol. It produces an irregular mortality pattern when inoculated into chicken embryos. The outstanding lesion seen in chicken embryos succumbing to this organism is severe pericarditis which is usually present in those dead after the seventh day postinoculation. This organism will withstand 56 C. for thirty minutes but not for one hour. It will remain viable for two to three weeks at 4 C. and for more than ten months at —40 C. It can be preserved by lyophilization. The organism does not produce an appreciable level of neutralizing antibodies in chickens, rabbits, or pigs. When the swine PPLO is inoculated intraperitoneally into pigs 6 weeks or less of age it produces severe fibrinous pericarditis, moderate fibrinous pleuritis and mild peritonitis. From 5 to 20 per cent of the inoculated pigs usually develop arthritis. Similar lesions produced by this organism occur in swine under field conditions.

The name *Mycoplasma hyorhinis* (n. sp.) is proposed for this organism.

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An Evaluation of Influenza in Midwestern Swine

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To THOSE familiar with disease problems of midwestern swine, it is apparent that there is a high incidence of respiratory diseases among these swine. From 50 to 70 per cent of lungs of swine at many packing plants show lesions indicating current or past respiratory disease. Most of the lesions are similar to or grossly indistinguishable from those described for swine influenza.[1]

Since 1949, we have tried to evaluate the extent of influenza among swine in relation to the incidence of respiratory disease as shown by lung lesions in the abattoir. The study has been primarily a serological one, employing hemagglutination-inhibition and serum-neutralization tests to indicate previous influenza infection. Vaccination studies were also included to evaluate possible control measures for lung infections in suckling pigs. The findings of these studies are herein reported.

MATERIALS AND METHODS

Serums.—The serum samples tested for influenza antibodies were from two sources: (1) selected groups of hogs slaughtered at a local packing plant and (2) brood gilts and sows from ten experiment station herds.

Serums obtained at the packing plant were from hogs shipped from ten known areas in Minnesota and Iowa. Samples were collected four times a year. Sampling among pigs by breeds was so designed that pigs from at least 1,000 farms were tested over a four-year period for evidence of past influenza infection.

Serums were obtained either in conjunction with experiment station brucellosis surveys or specifically for the influenza study. The samples were individually identified so that performance records could be correlated with serological findings.

Hemagglutination-Inhibition (HI) Test.—The HI test was a modification of the method of Salk[2] using chicken red blood cells (r.b.c.). Blood was collected weekly from 6 chickens into flasks containing 2 per cent sodium citrate. The chicken blood cells were thrice washed with buffered saline (0.005 M phosphate buffer in physiological saline at pH 7.1) by alternate centrifugation and decantation and checked for sedimentation characteristics. Those cells which settled normally were pooled to make a stock suspension of 30 per cent r.b.c.

Three different suspensions of r.b.c. were necessarily prepared daily for the HI test as performed in this laboratory. A 5 per cent suspension was used to absorb nonspecific hemagglutinins from swine serums, as is done with hamster serums,[3] and suspensions of 0.5 per cent and 0.25 per cent were prepared for the test proper. Since exact concentration was not necessary, the 5 per cent r.b.c. suspension was prepared from the 30 per cent r.b.c. suspension by dilution. The 0.25 per cent suspension was prepared from the 5 per cent suspension by further dilution with buffered saline to an equivalent turbidity of a McFarland No. 5 nephelometer standard in a Coleman spectrophotometer at 635 μ. The 0.5 per cent cell suspension was made from the 0.25 per cent suspension by centrifugation of the latter and restoration to half its original volume.

The HI test was done as follows: All serums were heated at 56 C. for ninety minutes. An initial serum dilution of 1:12.5 was made, using the 5 per cent r.b.c. suspension as diluent. After ten minutes, the r.b.c. were settled by centrifugation. The supernatant fluid was used to make serial twofold dilutions in r.b.c. suspensions, *i.e.*, 1:25, 1:50, and 1:100 for the HI antibody test. The first tube in the dilution series contained the 0.5 per cent r.b.c. suspension. Succeeding tubes in the series contained the 0.25 per cent r.b.c. suspension.

The viral antigens used in the HI test were swine influenza (Shope's strain 15), human influenza type A viruses (strains Weiss and PR–8), and human type B virus (strain Lee). All antigens were prepared by formolizing virus concentrated by Sharples centrifugation of infected extra-embryonic fluids from fertile hen's eggs. All antigens were used in a final concentration of 4 hemagglutination units, as described by Salk.[2] They were kept chilled in a refrigerator until just before addition to the cell-serum mixture. The cell-serum-antigen mixtures were held at 24–25 C. for ninety minutes before being read by a combined pattern and slip end point method. Complete inhibition was indicated by a round, confined concentration of cells in the bottom of the tubes which would slip freely when the tube was tilted.

Serum Neutralization Test.—An *in vitro* neutralization of virus was used to evaluate serum neutralizing (SN) antibodies. Five mice were used to test each serum-virus mixture for residual

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virus with evaluation of SN based on the intensity of disease in the test mice.

The influenza viruses were all mouse-egg adapted strains. Human type A strains PR–8 and Weiss, human type B strain Lee, and Shope's swine influenza strain 15 (S–15) were used. The S–15 virus was obtained from Dr. R. E. Shope of the Rockefeller Institute. All other strains were received from Dr. H. R. Cox of Lederle Laboratories. Viruses were maintained by allantoic inoculation of the virus into 10-day fertile hen's eggs. Virus for the SN test was obtained from portions of pooled infected allantoic fluids for each strain of virus harvested seventy-two hours after inoculation. These portions were stored in sealed glass ampules at —20 C.

The SN test was conducted as follows. One milliliter of serum, previously heated at 56 C. for one hour, was mixed with 1 ml. of infected allantoic fluid which had been diluted with hormone broth to contain approximately 2,000 times the amount of virus necessary to give an infectivity score of 2 (defined below). The serum-virus mixture was held in a 37 C. water bath for one hour. Five mice were given 0.05 ml. each of the serum-virus mixture intranasally while under light ether anesthesia. The material was instilled dropwise from a blunt 26-gauge needle attached to a 1-ml. tuberculin-type syringe. A control serum-virus mixture was similarly inoculated into mice.

Test mice in groups of 5 were kept in 6 by 7 inch glass jars with screen tops and were self-fed purina fox checkers and water. Jars were checked daily for dead mice and after ten days all living mice were destroyed and their lungs were observed for lesions.

The method for determining the intensity of infection in the mouse was essentially that of Horsfall [4] and of Lauffer and Miller.[5] Mice dying of influenza within the ten-day test period were each given a score of 4, and those that showed consolidation of ¾, ½, or ¼ of the lung area at necropsy on the tenth day were given scores of 3, 2, and 1, respectively. The final positive scores differed from those of Lauffer and Miller in that the total score for each test group was divided by four times the number of mice rather than by the total number of mice. This variation made it possible to represent infectivity as a percental number of the maximal possible score that would result from death of all inoculated mice.[6]

Vaccine.—The vaccine used in these studies was prepared by methods used for influenza vaccines for man. Five strains of influenza virus were used to make a polyvalent formolized vaccine.* The strains were S–15, human type A PR–8 and Weiss, human type A' FM–1, and human type B Lee. Virus was obtained from infected chicken embryo extra-embryonic fluids and concentrated by centrifugation so that each milliliter of vaccine contained 200 chicken cell agglutination units of each of the five strains of virus. This is approximately twice the strength used in vaccines for man. Two milliliter was adopted as the vaccine dosage, which is also twice that used in man.

Only sows or gilts to be bred for spring farrowing were vaccinated. Two to four weeks before breeding, a blood sample was taken from each gilt or sow, and vaccine was given intramuscularly to half of the prospective dams within a herd. In some herds, 1 or 2 additional doses of vaccine were injected, the second injection being given before breeding and the third midway in gestation. Blood samples were taken before each injection of vaccine and just before parturition so that antibody response could be determined by HI and SN tests.

PROCEDURES AND RESULTS

Table 1 shows the results of HI tests on

* We are indebted to Dr. Herald R. Cox, Lederle Laboratories Division of the American Cyanamid Co., Pearl River, N. Y., for vaccine and antigen.

TABLE 1—Hemagglutination-Inhibition (HI) Titers in Swine Serums for Influenza Viruses

Date of sample	No. serums tested	Percentage of serums showing HI titers for influenza viral antigens as indicated:*							
		Swine 15		PR–8		Weiss		Lee	
		1:100	1:200**	1:100	1:400**	1:100	1:400**	1:100	†
Oct. 1949	166	11	3	4	1	43	2	18	0
Dec. 1949	162	21	14	2	0	40	2	18	0
Feb. 1950	169	30	23	5	0	48	2	12	0
Apr. 1950	179	33	24	2	0	43	1	15	0
Oct. 1950	150	25	8	60	1	75	10	80	0
Dec. 1950	150	7	3	43	4	23	1	22	0
Feb. 1951	150	2	2	3	1	4	1	0	0
Apr. 1951	150	1	1	4	0	4	1	9	0
Apr. 1952	151	11	9	40	4	23	1	16	0
Oct. 1952	50	12	2	10	2	10	0	20	0
Dec. 1952	50	14	2	10	0	16	0	24	0
Feb. 1953	50	22	0	10	0	30	2	36	0
Apr. 1953	50	22	16	18	2	26	6	24	0
Total or average	1627	16	10	16	1	33	2	21	0

* Antigens were formolized virus from infected extra-embryonic chicken embryo fluids. Four chicken cell agglutination units were used as described by Salk.[2]

** Minimum titer acceptable as evidence of past infection by influenza.

† No relationship found between HI titer and capacity of swine serums to neutralize Lee virus (*see* table 3).

1,627 swine serums obtained by systematic sampling of swine on arrival at terminal markets. Hemagglutination-inhibition titers of 1:100 and over for S–15, PR–8, Weiss, and Lee viruses are shown in ordinary print. Titers at a level which is considered to be indicative of past encounters with the specific viruses are presented in bold-faced print. The minimal titers of 1:200 for S–15 virus and 1:400 for PR–8 and Weiss viruses were arrived at by comparing results from HI and SN tests on the same serums. Data will be considered later.

TABLE 2—Influence of Swine Influenza Hemagglutination-Inhibition (HI) Antibody on HI Titers to Human Viruses

Serums with S-15 HI titer	Serums showing HI titers 1:100 or greater to: PR–8 (No.)	PR–8 (%)	Weiss (No.)	Weiss (%)	Lee (No.)	Lee (%)
Less than 1:100 (1,369 samples)	215	16	397	30	260	19
More than 1:100 (258 samples)	42	16	136	53	77	30
Increased incidence (%)		0		77		58

During the four-year period under study, influenza among the swine from which the serums were taken (bold-faced type, table 1) was almost entirely of the S–15 type. The highest incidence of S–15 infection was 23–24 per cent in the 1949–1950 influenza season. The over-all incidence during the entire four years was 10 per cent. There was some evidence of infection with human type A strains PR–8 and Weiss, but such incidence was extremely low. Hemagglutination-inhibition titers in swine serums, at least in the test employed, are not a reliable indication of an infection with human type B virus (strain Lee). Hemagglutination-inhibition tests were also made for the FM–1 (human A′) virus. Titers of 1:5000 were common and all but one serum tested showed HI through dilutions of 1:400. No significance is attached to the FM–1 titers as they are apparently nonspecific.

Because of the high incidence of 1:100 HI titers when human influenza virus antigens were used, data were compiled to determine what influence the substances contributing to S–15 titers might have on titers to the other viruses (table 2).

It is apparent that S–15 titers did not influence those obtained with PR–8 antigen as the incidence of serums titered 1:100 was the same among those with and without S–15 titers of 1:100. An influence was evident, however, with an increased incidence of 77 per cent and 58 per cent, respectively, for Weiss and Lee antigens.

Serum-neutralizing tests with different viruses were also performed on many of the serums to evaluate the reliability of the HI test as an indicator of previous influenza infections. The results of such comparisons using S–15, PR–8, Weiss, and Lee viruses are given in chart 1. Serums with HI titers less than 1:100 are compared with those having HI titers of 1:100 or higher for their capacity to neutralize virus.

There was a difference in the protection afforded test mice with serums having S–15 HI titers over 1:100 and those with titers less than 1:100. An average infectivity score with these serums of 37 per cent indicates that many of them were capable of partially or completely neutralizing the 1,000 doses of virus given the mice. The infectivity score of 92 per cent for the serums less than 1:100 is near maximal because all mice challenged with 1,000 doses of S–15 virus rarely die in the ten-day test period, although their lungs may be over three-quarters consolidated.

The serums with PR–8 HI titers over

TABLE 3—Relationship of Hemagglutination-Inhibition Influenza Swine Serum Titers to Influenza Virus Neutralization Capacity of Serums

Virus strain	HI titer Over	HI titer Under	No. of serums tested	Serums neutralizing test influenza viruses* (No.)	Serums neutralizing test influenza viruses* (%)
S–15		1:100	220	3	1
	1:100	1:200	13	5	38
	1:200	1:400	14	10	71
	1:400		29	21	72
Totals			276	39	14
PR–8		1:100	153	2	1
	1:100	1:200	38	7	18
	1:200	1:400	21	4	19
	1:400		29	9	31
Totals			241	22	9
Weiss		1:100	160	5	4
	1:100	1:200	33	2	6
	1:200	1:400	51	7	14
	1:400		27	4	15
Totals			271	18	7
Lee		1:100	180	7	5
	1:100	1:200	25	1	4
	1:200	1:400	26	1	4
	1:400		21	0	0
Totals			252	9	4

* Serums which neutralized 1,000 test units of virus with infectivity scores less than 25 per cent (*See* Materials and Methods).

1:100 also showed some SN of PR–8 virus. The 58 per cent infectivity score with serums with titers over 1:100 is contrasted to an 84 per cent score with those serums

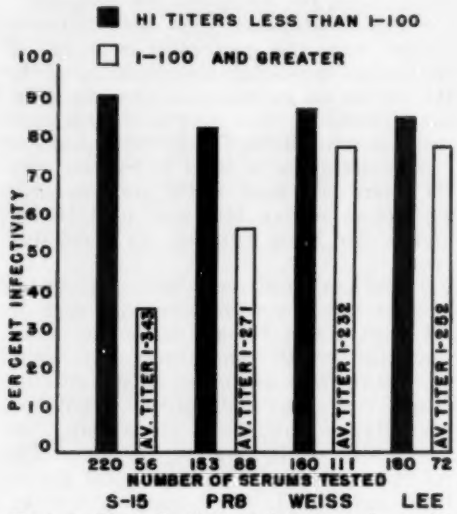

Chart 1—Neutralization of influenza virus with swine serum.

which had titers of less than 1:100. Some of the 153 serums without titers apparently had some influence in lowering the infectivity score, since scores of 94–96 per cent were generally obtained with control serums (table 4) used to neutralize 1,000 doses of PR–8 virus.

Only a slight lowering of infectivity scores was obtained with serums having HI titers 1:100 and over for Weiss and Lee viruses. A score of 78 per cent infectivity was obtained in tests with these serums. This score is not markedly lower than 89 per cent and 87 per cent for serums which had HI titers of less than 1:100 for Weiss and Lee viruses, respectively.

With the general observation (chart 1) that there was some relationship between HI titers of 1:100 and the ability of swine serums to neutralize influenza viruses, the data were also compiled to compare HI titers of 1:200, 1:400, and over 1:400 on relative percentage of neutralizing serums (table 3).

Two hundred twenty serums with HI titers of less than 1:100 to S–15 virus were subjected to SN tests. Of these, only three (1 %) were capable of neutralizing S–15 virus. As the HI titers increased, however, the percentage of serums with SN antibodies increased, with 71 per cent and 72 per cent, respectively, for serums of 1:200 and 1:400. The occurrence of SN in 14 per cent of the serums tested is in fair agreement with 10 per cent serums with HI titers of 1:200 and over (table 1).

To some extent, as HI titers in swine serums for PR–8 and Weiss increased, the percentage of serums showing SN activity increased. The relationship is so vague, however, that HI titers in swine serums to human viruses are of questionable significance. An incidence of 9 per cent (PR–8) and 7 per cent (Weiss) of influenza as shown by the SN test is considerably higher than the 1 per cent and 2 per cent incidence by the HI test using 1:400 titers as minimal.

There was no relationship between HI titers and SN activity to Lee virus. The percentage of serums showing SN activity was higher in the group of serums with HI

TABLE 4—Relative Neutralizing Capacity of Swine Serums for Different Viruses

Samples selected for neutralization of virus indicated	Per cent infectivity score* in mice to indicated test virus			
	S–15	PR–8	Weiss	Lee
Field samples				
S–15	3.6	17.3	35.0	71.4
PR–8	22.9	5.4	33.8	76.2
Weiss	6.7	6.8	2.9	58.8
Lee	45.0	11.7	56.9	11.9
Samples from vaccinated** swine				
S–15	10.3	25.3	29.1	77.8
PR–8	21.3	8.7	17.1	68.8
Weiss	25.8	17.1	7.1	75.4
Lee	70.8	71.7	71.7	18.3
Control serums	92.5	94.3	98.8	95.7

* Relative consolidation of mouse lungs, Score defined under *serum neutralization.*
** Selected serums from vaccination experiments (*See* table 6).

titers of less than 1:100 than with any other group.

The SN test was also used to evaluate possible immunological relationships of the viruses used for testing serums. Ninety-six serums were selected from among those tested for SN antibody (chart 1) and from vaccination studies (chart 2) for their capacity to neutralize specific viruses. Twelve samples were selected for each virus in each of two categories, *i.e.*, serum from swine which had not received influenza vaccine and from those that had. Only samples giving infectivity scores of less than 25 per cent were considered.

Each serum sample was tested concurrently for the specific virus it was capable of neutralizing and for the other three test viruses. Relative ability to neutralize the different viruses was thus determined. Results of these cross-neutralization studies are given in table 4.

An immunological relationship between type A viruses and the swine virus was apparent but no relationship existed between S–15 and Lee virus. The low infectivity score of 3.6 per cent for the S–15 virus is in marked contrast to the 71.4 per cent score obtained by attempting to neutralize Lee virus with the same serums. Intermediate scores for PR–8 (17.3%) and Weiss (35.0%) indicate ability of the 12 serums which neutralized S–15 virus readily to partially neutralize the two human type A influenza viruses. Samples showing low infectivity scores to PR–8 virus (PR–8 vs. Pr–8; score 5.4, table 4) also influence scores for S–15 and Weiss viruses. Viruses S–15 and PR–8 were influenced similarly in relation to each other and to Weiss virus, suggesting presence of common as well as specific SN antibodies. The 12 samples selected for SN activity on Weiss virus apparently contained considerable heterologous SN antibody as they had a pronounced influence on the infectivity scores of all test groups. This was also true to a lesser extent with the serums selected for activity against Lee virus.

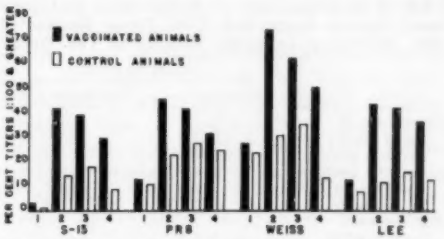

Chart 2—Comparison of hemagglutination-inhibition influenza titers in vaccinated and nonvaccinated swine; 1—prevaccination, 2—prebreeding, 3—midterm, 4—term.

When considering the SN capacity of serum from swine which received influenza vaccine, it must be remembered that antigens from all four test viruses were in the vaccine. The SN antibody response to swine influenza virus antigen (S–15) and the human type A virus antigens (PR–8 and Weiss) was moderate but definite. The response to type B virus antigen (Lee) was generally poor as is shown in the cross-neutralization tests.

Influenza Vaccination.—The main objective in vaccination of swine was to attempt to reduce mortalities among suckling pigs through transfer of specific lactoglobulins to the pigs via colostrum rather than to reduce or prevent disease in older animals.

TABLE 5—Relative Performance of Litters Farrowed by Control Dams and by Dams Vaccinated with Polyvalent Influenza Vaccine

	No. of litters	Pigs born alive	Pigs per litter	56-day mortality (%)	Pigs weaned per litter	Ave. weight 56-day	Statistical analyses*	
							Mortalities	Weaning weights
Early farrow								
Controls	13	109	8.4	50.5	4.2	24.2	$X^2 = 12.02$	$\bar{d} = 3.5$ lb.
							df = 1	s = 7.7 lb.
Vaccinates	14	116	8.3	28.5	5.9	27.7	P = less than 0.1%	df = 135
								P = less than 5%
Late farrow								
Controls	19	162	8.5	33.3	5.7	31.8	$X^2 = 6.5$	$\bar{d} = 3.1$ lb.
							df = 1	s = 9.6 lb.
Vaccinates	17	145	8.5	26.2	6.2	34.9	P = less than 1%	df = 212
								P = less than 5%
Totals	63	532	8.4	32.0	5.8	30.2		

* X^2 analysis by percentage mortality, Snedecor's Statistical Methods, 3rd ed., Iowa State College Press, Ames, p. 173; df is degrees of freedom and P is probability. Significance of weaning weight by sample size requirements using pooled standard deviation (s) and difference between means ($\bar{d}$). *Ibid.* p. 70.

It was for this reason that the dams were vaccinated before breeding.

The first vaccination trial was made on an experiment station herd with a long history of mediocre to poor performance of suckling pigs. The average loss among suckling pigs over a ten-year period was 26.3 per cent. In October, 1949, half of the gilts to be bred for the 1950 spring farrowing were given two injections consisting of 2 cc. of vaccine two weeks apart. The gilts were bred at first estrus after the second vaccination.

TABLE 6—Comparison of Performance of Litters from Control Dams and from Dams Vaccinated with Polyvalent Influenza Vaccine in Ten Herds.

	Litters farrowed by	
	Control dams	Vaccinated dams
Pigs farrowed		
No. of litters	247	233
No. born alive	1894	1785
No. born dead	154	138
Birth weight (ave.)	2.6	2.5
No. per litter	7.7	7.7
Pigs weaned		
Total weaned	1362	1283
No. per litter	5.5	5.5
Ave. weight per pig	32.7	32.9
Ave. weight per litter	180	181
Mortality (%)	28.1	28.1

Soon after the first litters were farrowed in March, 1950, a respiratory infection occurred among pigs within the barn. A moderate diarrhea accompanied the illness. The illness was not specifically diagnosed since no virus was isolated. Adult animals were not affected. Pigs farrowed by the vaccinated dams had a significant advantage over those farrowed by control dams (table 5) in both the early (March) and late (mid-April) farrowings. Pigs from vaccinated dams had higher livability and weight when weaned at fifty-six days.

To further test these findings, vaccination studies were extended for the 1951 farrowing season. One half of the dams in each of ten well-managed swine herds were vaccinated with formolized polyvalent influenza vaccine. It is apparent that the pigs farrowed by vaccinated dams had no advantage over those farrowed by control dams (table 6). Weaning weights and mortalities at fifty-six days were essentially the same.

Hemagglutination-inhibition titers of serums from the dams within these ten herds were similar at the time the first injection of vaccine was given (chart 2). The incidence of HI titers of 1:100 or higher increased in vaccinated dams as shown by serological tests on serums taken at intervals after vaccination. For the most part, titers persisted throughout gestation, suggesting that protection against influenza may have been transferred to the pigs at birth since it has been previously shown [7-9] that influenzal antibodies are transferred via the colostrum. The results in table 6, however, suggest that influenza was not a factor in the losses incurred.

DISCUSSION

The study reported here gives an estimate of the relative importance of the influenza viruses as a cause of respiratory disease among swine. Assuming that hemagglutination-inhibition (HI) titers of 1:200 for S–15 and 1:400 for PR–8 and Weiss viruses are indicative, the maximal incidence of influenza, as judged by this study, would be less than 15 per cent. This is a considerably lower incidence of respiratory disease than the lung lesions in swine coming to slaughter in the average packing plant would indicate, as 50 to 70 per cent of such lungs show evidence of some current or past respiratory disease.

It is apparent from the SN test for influenza antibodies, however, that the HI test employed in these studies is not a reliable test for certain antigenic types of virus. Hemagglutination-inhibition titers of 1:200 for S–15 viruses apparently is good evidence and 1:400 for PR–8 virus is fair evidence of previous influenza infection. Dilutions of greater than 1:400, however, would seem necessary for Weiss and Lee viruses, because there seems to be little or no association between the HI test and the SN test in swine serums. The incidence of influenza, considered only from the percentage of serums that neutralize influenza viruses, is less than 35 per cent without considering cross neutralization by some serums.

Failure of the HI test to truly indicate previous association with influenza viruses is almost likely due to nonspecific substances in the serum that give false positive HI titers. In the test used, all serums were washed with 5 per cent r.b.c. to remove some of the nonspecific substances but primarily to remove the nonspecific hemagglutinins. There are two other treatments commonly used in testing human serums to reduce the number of non-

specific positive titers. These are based on treatment of the r.b.c. with metaperiodate [10] or with a receptor-destroying enzyme derived from *Vibrio cholerae* filtrates.[11] Should further testing of swine serums be undertaken, use of a receptor-destroying enzyme to reduce nonspecific HI titers should be considered, since a test using this substance currently appears to be the one of choice between these two methods. Also, the use of guinea pig r.b.c. as suggested by Gulrajani [12] should be considered.

Since the influenza viruses appear to cause only a part of the respiratory disease among swine, it is of interest to consider other diseases which may be responsible for the lung changes observed in slaughtered swine. In the last few years, a disease or diseases which show gross pathological changes of the lungs indistinguishable from swine influenza has been reported from many different countries.[13–20] This disease has become known as virus pneumonia of pigs (VPP), and since it has been reported in swine in Saskatchewan [20] it is possible that VPP is common in swine in the United States. In fact, Beveridge, who with Betts [21] reports a high incidence of VPP in British swine, has indicated that it is as common in swine in Ohio as in England.[22] This statement is based on observations made while Beveridge was a visiting professor at Ohio State University in 1953. Virus pneumonia of pigs characteristically results in poor performance of swine [23] and because of its economic importance research directed toward determination of its incidence among midwestern swine and possible control measures should be undertaken.

Failure of vaccination of dams before breeding to lower mortalities among the pigs they farrowed would appear to be due to other factors than lack of immunological response to vaccination. It has been quite well established that injection of either live [7,24,25] or formolized [26–30] influenza viruses stimulates immunity to influenza in swine. The results in our studies in 1950 (table 5) indicate that vaccination may be beneficial when there is infection among very young pigs.

There has been a tendency among swine producers to accept mortalities of 25 to 30 per cent among suckling swine as inherent in swine rearing. Were this loss necessary, we could accept the 28.1 per cent losses of suckling pigs incurred in the vaccination experiments as unavoidable and look elsewhere for areas of fruitful study. There is good evidence, however, that such losses need not occur. One large, well-managed herd under study by this laboratory during the past seven years has lost less than 7.5 per cent of its suckling pigs (0 to 56 days—average weight 42 lb.) for the last three successive years. Excellence of performance among postweanling pigs with average weights over 200 lb. in 140 days is partially attributed to good health during the suckling period.

Further evidence that high motalities among suckling pigs need not occur is afforded by studies in which pigs were obtained by hysterectomy and reared in isolation on modified cow's milk.[31] Because these pigs do not receive colostrum, they are possibly not as well off nutritionally as pigs which nurse their dams. Losses among these artificially reared pigs, however, have been less than 10 per cent, which is in marked contrast to the loss of 25 to 30 per cent, as experienced by producers in general. High livability is attributed to strict limitation of diseases capable of killing the suckling pigs.

Evaluation of the data presented points toward influenza as a chance disease among suckling swine with heavy losses occurring only when very young pigs are attacked. The usual losses of 25 to 30 per cent would appear to be due mainly to other causes—most likely as-yet-unidentified diseases of young pigs. Because of the high incidence of respiratory infection among older swine which is not attributable to infection by influenza, other respiratory diseases of young swine should be studied as potential contributors to baby pig mortalities. Virus pneumonia of pigs would seem to be one disease which could logically be studied in this respect because of its apparent prevalence, long persistence in the host, which is not characteristic of influenza,[32,33] and poor stimulation of immunity.

Summary

A study of influenza in swine from 1949–1953 serves as the basis for this paper. More than 2,000 swine serums have been tested for presence of hemagglutination-inhibition (HI) or serum-neutralization (SN) antibodies or for both as produced by natural infections or by injection of formolized polyvalent influenza vaccine.

The HI test used was found to be indicative of previous infection by influenza when swine influenza, strain S–15, and type A human influenza, strain PR–8, antigens were used. It was not indicative of Weiss (human type A), FM–1 (human type A′), or Lee (human type B) antigens, probably because nonspecific substances gave false positive titers.

Serum-neutralization tests on representative serums suggested an immunological similarity of the S–15 swine influenza virus and the two human type A viruses. The closest relationship was between S–15 and PR–8.

Vaccination of dams before breeding with formolized polyvalent influenza vaccine did not greatly reduce mortalities among suckling pigs farrowed by these dams. It appears that benefit was derived, however, in a single herd during one farrowing season. Failure of the vaccine to reduce mortalities is attributed to lack of occurrence of influenza in young pigs within the herds vaccinated rather than to the inability of the vaccine to produce antibodies.

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The Effect of Antibiotics upon the Motility of Isolated Intestinal Sections of Rabbits and Swine

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NUMEROUS effects of antibiotics have been described in the literature, among them a wide variety of influences in the intestinal tract of animals.

Some of these beneficial effects are believed to be due to suppression of those organisms in the intestinal flora that compete with the host for dietary components,[1] the possibility of increasing food consumption,[2] the control of a low level of disease that would have an adverse influence on growth,[3] and the control of scouring.[4] However, the administration of 600 mg. per day of an antibiotic caused beef cattle to develop diarrhea.[5]

This paper presents the results of an experiment designed to investigate the action of antibiotics on the motility of isolated intestinal sections of the rabbit and of swine, independent of various physiological influences and of its contents.

EXPERIMENTAL DATA

Pigs weighing 3 to 45 lb. and rabbits weighing 3 to 6 lb. were used. These animals had not been fed antibiotics at any time and did not show signs of digestive disturbances at the time they were killed. Their abdomens were promptly incised, about 6 to 10 inches of jejunum was removed and the contents were rinsed out with water, using a pipette placed in one end of the section. A piece of intestine about 1 inch in length was cut off and mounted in a chamber. The chamber (200- or 400-ml.) was partially immersed in a water bath whose temperature was kept constant by a heating coil.

Tyrode's solution, used as the muscle bath, was prepared the day before the test and warmed to the bath temperature before the muscle was placed in it. A slow stream of air entered the chamber through a glass tube bent at the bottom in such a way as to allow for the attachment of the muscle by a pin and thread. A pin through the other end of the muscle was attached by a thread to the end of a lever arm that contacted the continuous feed, ink writing kymograph. The timing mechanism was adjusted to record periods of six seconds.

Procaine penicillin,* chlortetracycline,** dihydrostreptomycin,† procaine hydrochloride,‡ potassium penicillin,‖ or bacitracin§ were dissolved in distilled water or Tyrode's solution and added to the bath at various concentrations (tables 1, 2, 3, and 4). Each concentration of procaine or antibiotic preparation was added to the bath at five-minute intervals. The muscle bath containing the added material was replaced by fresh Tyrode's solution after every trial. This afforded an opportunity to observe the possible recovery of motility by the muscle following the effects of the procaine or antibiotic preparation.

RESULTS

Procaine penicillin when added to the bath produced a decrease in the amplitude of rhythmic contractions (table 1 and fig. 1). It appeared to have a cumulative effect on the intestinal motility, for each addition slightly decreased the amplitude of contraction. This effect was particularly evident in trials 5 and 6. The decrease in amplitude appeared to be proportional to the amount of procaine penicillin added at any one time. A high concentration (trial 7) completely inhibited muscle motility and caused relaxation of the muscle. In all trials, the muscle returned to its initial level of activity when placed in a fresh Tyrode's solution.

The addition of procaine penicillin to the bath surrounding isolated swine intestinal sections (table 2 and fig. 2, trials 16 and 18) had only a slight relaxing effect with additional procaine penicillin being ineffective except to possibly cause the incidence of tonus waves. In most of the

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* Penicillin G procaine, crystalline in aqueous suspension, 300,000 units per milliliter; Chas. Pfizer & Co., Inc.

** Aureomycin hydrochloride, crystalline; Lederle Laboratories.

† Dihydrostreptomycin sulfate, crystalline; Chas. Pfizer & Co., Inc.

‡ U.S.P. XIII; Mallinckrodt.

‖ Crystalline penicillin G, potassium salt; Commercial Solvents Corp.

§ Bacitracin powder, Commercial Solvents Corp.

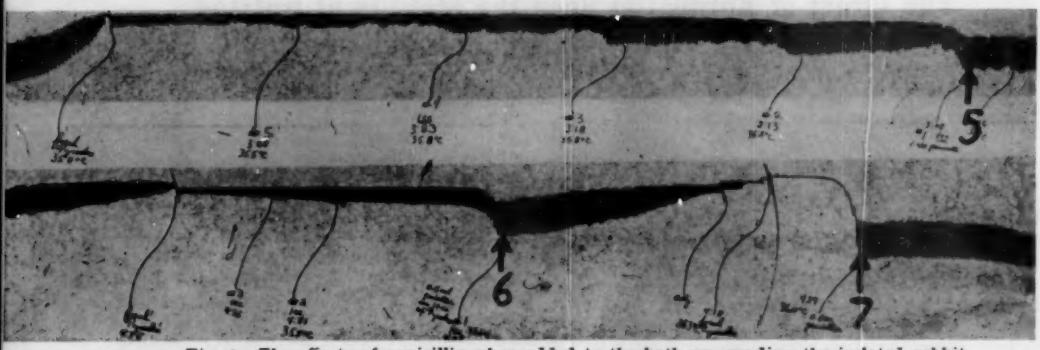

Fig. 1—The effects of penicillin when added to the bath surrounding the isolated rabbit intestinal sections (trials 5, 6, 7).

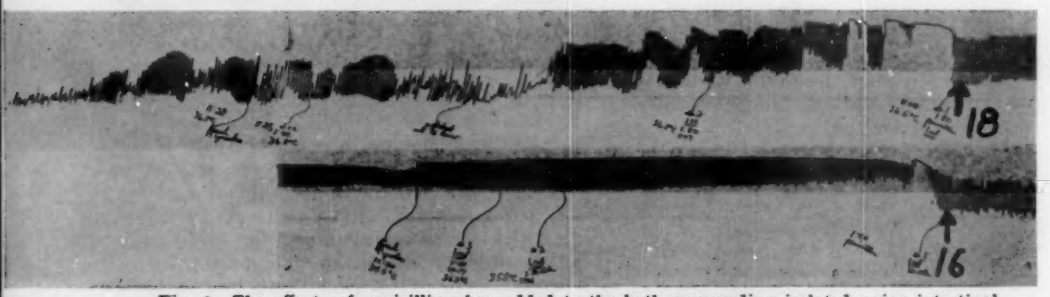

Fig. 2—The effects of penicillin when added to the bath surrounding isolated swine intestinal sections (trials 16, 18).

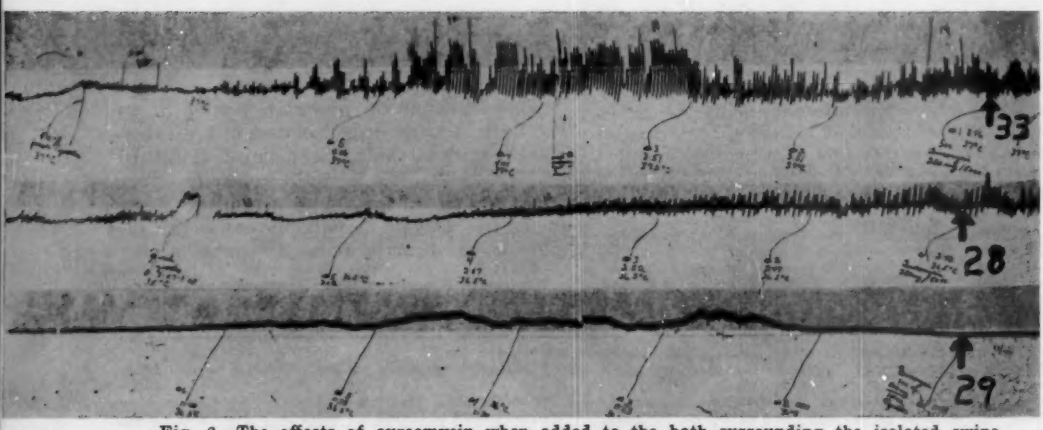

Fig. 3—The effects of aureomycin when added to the bath surrounding the isolated swine intestinal sections (trials 28, 29, 33).

TABLE 1—The Rate of Addition and Final Concentration of Procaine Penicillin, Procaine or Potassium Penicillin Added to the Bath Surrounding Rabbit Intestinal Sections

Trial	Units/ml. added/ 5 min.	Ml. added	Final concentration/ 100 ml.	Results
		PROCAINE PENICILLIN		
	units		units	
1	5,000	2	6,452	Decreased amplitude, relaxed muscle.
2	4,700	6	8,057	Decreased amplitude.
	18,800	1	13,429	Decreased amplitude and motility.
3	21,000	1	14,789	Inhibited motility, relaxed muscle.
4	4,700	8	10,743	Decreased amplitude.
	14,100	3	22,829	Decreased amplitude.
5	7,500	5	26,786	Decreased amplitude.
6	15,000	3	30,000	Decreased amplitude.
7	150,000	1	100,000	Inhibited motility, muscle relaxed.
		PROCAINE		
	mg.		mg.	
8	2.0	1	1.3	Immediate relaxation.
9	0.4	5	1.3	Decreased amplitude, accumulative effect.
10	2.0	1	1.5	Immediate relaxation, decreased amplitude.
		POTASSIUM PENICILLIN		
	units		units	
11	5,000	2	6,993	Tonus wave disappeared.
	5,000	3	17,483	Amplitude temporarily increased.
	20,000	1	31,469	
12	10,000	6	38,961	No apparent effect.
	30,000	1	58,442	No apparent effect.
13	150,000	1	109,489	Immediate slight contraction.
14	70,000	3	140,000	Immediate slight contraction.
	70,000	1	186,667	Decreased amplitude.

trials, procaine penicillin inhibited the motility of the sections and caused a slight relaxation of the muscle. Swine intestinal sections responded differently to procaine penicillin than did the rabbit sections; there seemed to be no cumulative effect and the sections seemed to be unaffected by additional procaine penicillin following the initial response. The swine intestinal section became nonmotile in 2,586 units of procaine penicillin per 100 ml. of bath whereas the rabbit intestinal motility was only slightly affected by 10,000 units and motility was not stopped until a concentration of 100,000 units was used.

The question of the effect of procaine upon the motility of the isolated intestinal strips becomes important in determining the effect of the penicillin. The addition of procaine hydrochloride in trials 9 and 10 affected the muscle in a manner similar to that which occurred in trials 5 and 1, respectively. Thus it appears from these data that the response of the muscle to procaine penicillin was due to a very large extent to the procaine present in the preparation.

In order to eliminate the procaine effect and evaluate the effect of penicillin upon the intestinal motility, the potassium salt of penicillin was added to the muscle bath. In most trials there was no apparent effect of the potassium salt of penicillin, although at high concentrations slight contraction of the muscle occurred.

When chlortetracycline was added to the bath surrounding isolated rabbit intestinal sections (table 3) it decreased the amplitude of rhythmic contractions and inhibited the motility of the muscle.

When chlortetracycline was added to the bath surrounding isolated swine intestinal sections (table 3 and fig. 3, trials 28, 29, 33), it inhibited motility of the muscle, although the initial responses to the added antibiotic varied. In trial 28, chlortetracycline caused a decrease in amplitude; in trial 29, it caused over-all lengthening of

TABLE 2—The Rate of Addition and Final Concentration of Procaine Penicillin, Procaine or Potassium Penicillin Added to the Bath Surrounding Swine Intestinal Sections

Trial	Units/ml. added/ 5 min.	Ml. added	Final concentration/ 100 ml.	Results
		PROCAINE PENICILLIN		
	units		units	
15	3,750	1	2,586	Inhibited motility, muscle relaxed.
16	7,500	1	5,357	Inhibited motility, muscle relaxed.
	3,750	1		Slight decrease in amplitude.
	7,500	1	13,393	
17	7,500	3	15,734	Decreased amplitude.
18	3,750	1	2,641	Inhibited motility, muscle relaxed.
	3,750	1		
	15,000	1	15,845	Erratic.
19	30,000	2	42,850	Accumulative effect, decreased number of contractions per unit of time.
		PROCAINE		
	mg.		mg.	
20	12	2	16.0	Decreased amplitude.
		POTASSIUM PENICILLIN		
	units		units	
21	2,500	5	8,620	No effect.
	5,000	5	25,860	No effect.
22	10,000	5	33,333	No effect.
23	25,000	5	78,120	No effect.
	50,000	1	109,300	No effect.
	500,000	1	421,800	Slight decrease in amplitude.

the muscle; and in trial 33, the amplitude of rhythmic contractions were increased followed by inhibition of motility. In the latter trial, the muscle did not recover to the level of activity it showed prior to the addition of the antibiotic.

When dihydrostreptomycin was added to the bath for intestinal sections (table 4), there appeared to be no cumulative effect upon the motility of rabbit muscle, with a concentration of 150 mg. of dihydrostreptomycin per 100 ml. of Tyrode's solution being required to affect the amplitude of the rhythmic contractions. However, the motility of swine intestinal sections was more sensitive, the dihydrostreptomycin inhibiting motility and producing relaxation. Considered on a weight basis procaine penicillin (trial 7) had a much greater effect upon the muscle motility than did dihydrostreptomycin.

Bacitracin was used in two trials. It had no effect upon the motility of the isolated rabbit intestinal sections at final bath concentrations of 64 mg./100 ml. or at 72 mg./100 ml.

TABLE 3—The Rate of Addition and Final Concentration of Aureomycin Added to the Bath Surrounding Rabbit and Swine Intestinal Sections

Trial	Units/ml. added/ 5 min.	Ml. added	Final concentration/ 100 ml.	Results
		RABBIT INTESTINAL SECTIONS		
	mg.		mg.	
24	5	3	4.3	No apparent effect.
25	5	5	7.1	Decreased amplitude.
26	5	4	1.3	Decreased amplitude.
	20	1	2.6	Inhibitory motility.
27	20	1	1.3	Inhibitory motility.
		PIG INTESTINAL SECTIONS		
	mg.		mg.	
28	5	5	6.9	Decreased amplitude.
29	5	6	7.7	Initial slight increase in amplitude, muscle relaxed.
30	5	1	3.8	Temporarily inhibited motility.
	5	1	7.5	Temporarily inhibited motility.
	5	1	12.8	Inhibited motility, but recovered in fresh Tyrode's solution.
31	5	5	17.2	Decreased amplitude.
32	10	5	34.0	Accumulative effect, decreased amplitude and number contraction per unit of time.
33	10	1	6.8	Inhibited motility.
34	20	4	51.9	Accumulative effect, inhibited motility, relaxed muscle.
35	25	5	32.0	Initial increase in amplitude followed by decrease of amplitude and number of contractions per unit of time.

TABLE 4—The Rate of Addition and Final Concentration of Dihydrostreptomycin Added to the Bath Surrounding Rabbit and Swine Intestinal Sections

Trial	Units/ml. added/ 5 min.	Ml. added	Final concentration/ 100 ml.	Results
		RABBIT INTESTINAL SECTIONS		
	mg.		mg.	
36	5	5	7.1	No effect.
	20	5	35.7	Slight decrease in number of contractions per unit of time.
	500	1	150.0	Decrease amplitude and number of contractions per unit of time, no effect on over-all length of muscle.
		SWINE INTESTINAL SECTIONS		
	mg.		mg.	
37	5	3	10.3	Decreased amplitude.
38	25	1	16.1	Inhibited motility, relaxed muscle.
39	25	1	18.5	Inhibited motility, relaxed muscle.
40	5	3	10.0	No apparent effect.
	25	1	26.7	Slight decrease in amplitude, slight relaxation of muscle.
41	5	6	20.0	Accumulative effect, decreased motility, slight relaxation.
	25	1	36.7	Decreased motility.

DISCUSSION

The effects of the antibiotic preparations used in this study were in general decreased amplitude of rhythmic contractions or inhibition of motility of the isolated rabbit and swine intestinal sections. Relaxation or over-all lengthening of the rabbit muscle strips occurred only when procaine hydrochloride or procaine penicillin was added to the bath, whereas the swine muscle relaxed in the presence of all preparations tested except potassium penicillin. Procaine penicillin produced the most marked effects and at comparatively low concentrations. Procaine penicillin decreased the amplitude of rhythmic contractions and, at higher concentrations, decreased motility and caused relaxation of the muscle. However, the addition of procaine hydrochloride to the muscle bath produced responses similar to those produced by procaine penicillin. The potassium salt of penicillin usually caused an immediate slight contraction with subsequent recovery. The differences in responses were probably due to the presence of procaine or potassium in the antibiotic

preparation, which indicates that penicillin itself probably does not affect the motility of isolated muscle.

Hillerman and co-workers [6] reported the feeding of procaine penicillin (50 mg./kg. ration) to turkeys slowed the rate of feed passage through the digestive tract by an average of eighteen minutes as compared to those not getting the antibiotic, but this difference was of doubtful significance. They found age had a greater effect than did the penicillin.

The information obtained in the present studies indicates some antibiotic preparations act by decreasing the amplitude of rhythmic contractions and decreasing motility of the intestinal section. If the intestine of the intact animal is influenced by antibiotic preparations in the same way as the isolated intestinal sections, some antibiotic preparations could slow the rate of the passage of food materials through the digestive tract.

SUMMARY

The effects of procaine hydrochloride and of the antibiotic preparations: chlortetracycline, procaine penicillin, potassium penicillin, dihydrostreptomycin, and bacitracin upon the motility of isolated intestinal sections of rabbits and swine have been investigated. Procaine penicillin and procaine hydrochloride decreased the amplitude of rhythmic contractions and, at high concentrations, decreased the motility and relaxed the muscle. These two preparations appeared to have a cumulative effect upon the motility of the rabbit muscle. Potassium penicillin had very little effect upon motility except for the production of slight transient contractions. Penicillin independent of procaine or potassium probably does not affect the motility of isolated intestinal muscle of rabbits or swine. Chlortetracycline decreased the amplitude of the rhythmic contractions and inhibited the motility of rabbit muscle. It had a varied effect upon the motility of swine muscle but, in general, decreased amplitude of contractions followed by inhibition of motility. Dihydrostreptomycin decreased the amplitude of contraction, inhibited motility, and relaxed swine muscle. Bacitracin appeared to have negligible effects upon isolated muscle. Swine muscle and rabbit muscle differed in their response to antibiotic preparations. In general, higher concentrations of the preparations were required to produce responses in the rabbit muscle than in the swine muscle.

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An Experiment on the Cumulative Efficacy of Phenothiazine-Salt Mixture Against Nematodirus and Haemonchus in Lambs During a Second Grazing Season

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THE WRITERS [1] previously reported the results of two similar experiments, which showed that a 1:9 phenothiazine-salt mixture, administered free-choice, gave a considerable measure of control of mixed infections of *Nematodirus spathiger* and *Haemonchus contortus* acquired by lambs grazed on heavily contaminated pasture. The medicated and unmedicated lambs in both previous experiments, carried out in successive years, were grazed on separate pastures of as nearly equal larval contamination at the start of each of the two experiments as it was possible to arrange; thus, the second experiment was a replication of the first. These results gave no information on the cumulative advantage of this regimen of medication such as might accrue when lambs are continued on the same pasture over a second, successive grazing season or a considerable part thereof. Therefore, such an experiment was performed with groups of lambs similar to those previously employed,[1] except that the medicated lambs were grazed on the same contaminated pasture that was used by the medicated lambs the previous year, and the unmedicated lambs grazed the same contaminated pasture that was used by the unmedicated lambs the year before. A third comparable group of lambs was grazed at the same time on a relatively clean pasture. As the previously reported replicated experiments [1] were referred to as "first and second trials," the experiment reported herein will be referred to as the "third trial."

The pertinent literature has been cited previously [1] except for recent reports of Price, Hardy, and Boughton [2,3] which showed that a 1:9 phenothiazine-salt mixture satisfactorily controlled *H. contortus* in range sheep in Texas over a period of several years.

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EXPERIMENTAL PROCEDURE

The general procedures, pastures, and pasture arrangements were the same as those of the second trial,[1] except that observations were continued for only twelve weeks instead of twenty-four weeks. The pastures were unoccupied from late October following the second trial to early May of the next year, when 12 parasite-free Shropshire lambs (approximately 2 months of age) were divided into three comparable groups of 4 lambs each and confined on the pastures as in previous trials. Throughout the experimental period, the lambs of group A on contaminated pasture (¼ acre) were given access to a 1:9 phenothiazine and salt mixture, while those of group B on contaminated pasture (¼ acre) and group C on clean pasture (¼ acre) were offered salt only. The average daily consumption of phenothiazine per lamb was 0.226 Gm., or 2.26 Gm. of the mixture. Each of the lambs of group B and of group C consumed, on the average, 4.46 and 4.60 Gm., respectively, of plain salt per day. The daily consumption of the medicated mixture was higher during the first and second trials, 5.04 and 2.82 Gm., respectively, than in the third trial. Biweekly wool analyses of staple length and mean fiber diameter of clipped lock samples, and terminal analyses of individual lamb fleeces, including grease and clean scoured weights, were obtained. Carcass grades of the surviving lambs were determined prior to necropsy.*

Animals were observed daily for clinical manifestations of parasitism. At biweekly intervals, individual weights were taken and differential fecal egg counts and packed red cell volume (hematocrit) determinations were made. Total worm counts were made postmortem by methods previously described.[1]

RESULTS

Once substantial infections were established in the lambs grazing the contaminated pastures, the lambs on the phenothiazine and salt mixture (plate 1, fig. 3) had lower fecal egg counts of *N. spathiger* and *H. contortus* throughout most of the experimental period than the comparable

* The writers express their appreciation to Dr. T. D. Watkins Jr., Mrs. Mary Hourihan, and Mr. F. E. White for making the wool analyses, and to Messers. D. A. Spencer, C. G. Potts, and R. L. Clark for evaluation of carcass grades, all of the Animal and Poultry Husbandry Research Branch, Agricultural Research Service, U.S.D.A.

lambs on salt only (plate 1, fig. 4); this was particularly noticeable during the latter weeks of the experiment. As expected from the previous trials, there was a greater difference between the two groups in the egg counts of *H. contortus* than in those of *N. spathiger*. The early peak of *N. spathiger* egg counts in lambs on both pastures A and B (plate 1, fig. 3 and 4) demonstrated that large numbers of larvae of this species had survived over winter on these pastures. Initial egg counts of *N. spathiger* of both medicated and unmedicated lambs were near or slightly over 1,000 per gram of feces, such counts being indicative of massive infections of this species. In contrast, the peak egg counts of *H. contortus* occurred much later in the course of the experiment, as a result of the low over-winter survival of larvae of this

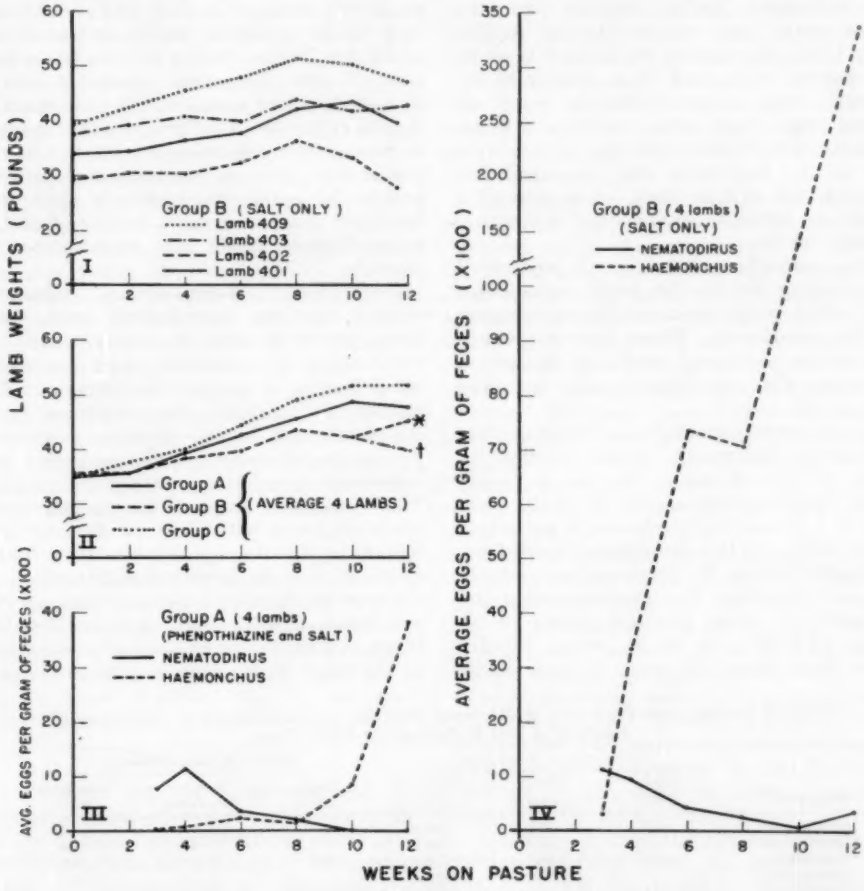

PLATE I

Selected weight and fecal egg count data; group A—medicated lambs on contaminated pasture; Group B—unmedicated lambs on contaminated pasture; group C—unmedicated lambs on relatively clean pasture.

Fig. 1—Weight data of unmedicated lambs showing individual variations over experimental period.

Fig. 2—Average weight gains of three groups of lambs (* average of terminal weights of 2 lambs surviving to end of experiment from pasture B; † average of terminal weights of 4 lambs from pasture B, including the 2 lambs which died during the twelfth week).

Fig. 3—Average fecal egg counts of medicated lambs.

Fig. 4—Average fecal egg counts of unmedicated lambs on contaminated pasture.

species, which necessitated recontamination of the pasture before substantial infections became established in the lambs. In the unmedicated lambs (pasture B), the Nematodirus egg counts reached an early peak and decreased gradually to the tenth week when an exacerbation of the infection in 2 lambs resulted in an increase in the average egg output. The Haemonchus egg counts in these lambs rose sharply to an extremely high level by the twelfth week. In the medicated lambs (pasture A), the Nematodirus egg counts started moderately high, increased to the highest peak by the fourth week, and then gradually decreased until negative counts were obtained the tenth and twelfth weeks, whereas the Haemonchus egg counts were low in the beginning and remained low through the eighth week, then showed a moderate increase during the tenth and twelfth weeks.

Two unmedicated lambs on pasture B died during the twelfth week and another died while being shorn at the termination of the experiment. There were no deaths among the medicated lambs on pasture A or among the unmedicated lambs on clean pasture C.

As in previous trials,[1] there were marked differences in weight gains among the three groups of lambs. Group A (medicated) lambs gained a total of 52 lb. (ave., 13.0 lb.) during the twelve-week period, or 34 lb. (ave., 8.5 lb.) more than those of unmedicated group B, which gained only 18 lb. (ave., 4.5 lb.). The unmedicated group C lambs on clean pasture gained 68 lb. (ave., 17.0 lb.), or 16 lb. (ave., 4.0 lb.) more than those of group A and 50 lb. (ave., 12.5 lb.) more than those of group B.

Scouring occurred among the unmedicated lambs on contaminated pasture B, in lamb 409 for a short period during the second week, and in lamb 403 during the last three days of the experiment. There was no evidence of diarrhea in lambs on pastures A and C.

Erythrocyte levels were comparable to those observed in previous trials.[1] The average hematocrit decreased slightly from an initial average level of 32.4 to 28.0 per cent in the medicated lambs on pasture A, and from 35.6 to 30.5 in the lambs on pasture C, but decreased markedly,—from 35.6 to 10.5 per cent—in the unmedicated lambs on pasture B. Thus, a slight decline in packed red cell volume occurred in the medicated lambs on contaminated pasture and in the lambs on a relatively clean pasture, but a severe anemia developed in the nonmedicated lambs on contaminated pasture.

Postmortem worm counts (table 1) showed that the unmedicated lambs harbored 20 times more *H. contortus* and 16 times more *N. spathiger* than the medicated lambs. A greater percentage of immature *N. spathiger* was recovered from the medicated lambs, whereas a greater percentage of immature *H. contortus* was recovered from the unmedicated lambs. This consistent finding during all three trials conforms with the view that medication is ineffective against immature parasites and may be largely due, therefore, to the over-wintering of Nematodirus, on the one hand, and to the rapid build-up of Haemonchus, in the absence of medication, on the other. Very small numbers of *Stron-*

TABLE 1—Parasites Recovered at Necropsy from All Lambs Exposed to Contaminated Pastures A and B During the Third Trial

	Pasture A (medicated)			Pasture B (unmedicated)		
Parasites*	Lamb (No.)	Worms recovered	Immature (%)	Lamb (No.)	Worms recovered	Immature (%)
Nematodirus	407	900	100.0	401	21,200	78.5
Haemonchus		1,400	48.5		47,000	75.0
Nematodirus	416	1,400	50.0	402	30,600	50.1
Haemonchus		2,700	50.0		19,500	60.7
Nematodirus	421	1,950	100.0	403	30,300	51.6
Haemonchus		1,300	38.4		86,200	87.5
Nematodirus	422	850	78.8	409	400	100.0
Haemonchus		2,800	28.5		9,950	81.9
	Totals			Totals		
Nematodirus		5,100	82.3		82,500	58.2
Haemonchus		8,200	40.6		162,650	80.3
Nematodirus	Ratio medicated to unmedicated			1.....to.....16		
Haemonchus				1.....to.....20		

* Insignificant numbers of parasites other than those listed also recovered but not tabulated.

gyloides papillosus, Trichostronglyus spp., and *Ostertagia circumcincta* were present, also, but these were considered to be of little or of no significance.

Lamb 409 of the unmedicated group (table 1) had a disproportionately low worm count at necropsy, the numbers of *N. spathiger* (400) and *H. contortus* (9,950) being comparable to those recovered from the medicated lambs. Furthermore, all specimens of the small number of Nematodirus from this lamb were immature, as were over 80 per cent of the Haemonchus. It is evident that lamb 409, unaided by medication, acquired a strong resistance to reinfection to both species of parasites. This lamb, however, did not escape the effects of exposure to the heavily contaminated pasture since it had relatively high maximum fecal egg counts—390 Nematodirus eggs per gram of feces the fourth week and 7,920 Haemonchus eggs per gram the sixth week of the experiment—a terminal hematocrit of 14 (about 40% of normal), a poor weight gain, and a carcass grade of "common" when the experiment was terminated.

The antemortem carcass grades of the several animals at the end of the experiment were as follows: group A, 1 common, 3 medium, averaging medium; group B, 2 common, 2 cull, averaging cull; group C, 1 good, 2 medium, and 1 common, averaging medium.

Wool analyses during the course of the experiment showed no significant differences among the three groups of lambs. This was probably due to the experiment being conducted over a relatively short period of twelve weeks, which may have been too short a period for the development of significant changes in the wool.

Discussion

The results confirm those obtained in previous trials. Moreover, they suggest that the regimen of medication, which derives its beneficial action largely from progressive decontamination of pastures, affords the same practical and progressive control of *N. spathiger* as it does of several other economically important gastrointestinal parasites of sheep. This cumulative efficacy of phenothiazine-salt mixture against Nematodirus and Haemonchus is shown by improvement of control in the third trial over the second trial in respect to worm burdens, egg counts, and lamb weights. Therefore, any advantage that may have accrued initially to the medicated lambs in this trial is presumed to have been due to the disinfestation of the pasture which presumably resulted from the fact that lambs grazing this pasture the previous season had access to phenothiazine and salt.

Comparative total worm-count data show that while excellent control of *H. contortus* and *N. spathiger* was achieved both years in the medicated lambs, better control of *N. spathiger* was attained in the current experiment. Moreover, the difference in worm burdens of treated and untreated lambs is considerably weighted by the low *H. contortus* count of resistant, untreated lamb 409.

Egg counts during the two trials also showed that better control of Nematodirus was achieved in the third trial. An initial difference in Nematodirus egg counts in these trials may have been caused by a greater over-winter survival of larvae prior to the third trial which may have resulted from the shorter interval between removal of the lambs and replacing others on the pasture. The latter was fallow for eight and one-half months before the second trial, and only six months before the third. Haemonchus egg counts were slightly, but not significantly, higher for the medicated lambs in the third trial.

Improved parasite control is also shown by greater weight gains of medicated lambs while lambs on clean pasture during these trials gained 16.0 lb. and 47.5 lb. more than the medicated lambs for the 12-week and 24-week periods, respectively.

The terminal carcass grades of the lambs of groups A and C in this and the previous trial were at least one complete grade above the lambs of group B, and in both trials the average grade of lambs of group C was slightly higher than those of group A. All three groups averaged a full grade lower in the third trial than in the second trial. This was due, possibly, to differences in weather, quality, and quantity of forage, infestation of pastures, and other uncontrollable variables. Five of 8 animals from pasture B died during the two trials. There were no deaths of animals maintained on pastures A or C.

There was apparently an acquired resistance to re-infection in one lamb (409)

on pasture B during the third trial. This lamb had a smaller terminal burden of Nematodirus (all immature) than any other lamb. It also harbored relatively small numbers of Haemonchus. However, it suffered serious parasitism during the course of the experiment and had a terminal carcass grade of common. The record of this lamb shows that "self-cure" probably occurred.

SUMMARY

The continued use of free-choice medication with phenothiazine and salt during successive grazing seasons has been shown to be advantageous in controlling combined natural Haemonchus and Nematodirus infections in lambs. Medicated lambs, as compared to nonmedicated controls, showed lower egg counts, fewer total worms, greater gains, better condition, and higher blood levels during the two trials. The lambs benefited more from the medication than comparable lambs of the previous season, indicating that the regimen achieves progressive decontamination of pastures in successive seasons. All treated lambs survived the exposure whereas 5 of 8 untreated lambs succumbed during the two trials. Comparable lambs, unexposed to gross parasitism and untreated, made the best gains during the two successive years.

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Effect of Phenothiazine on Conception Rate in Sheep

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DESPITE widespread use of phenothiazine in the control of parasites in sheep, the available literature mentions no attempt to evaluate a possible effect of this compound on the conception rate of these animals. Evidence that there is an adverse effect is inconclusive, if not entirely lacking.

We are indebted, however, to Dr. A. O. Foster[2] for citing two unpublished instances in which the question of such effect had been raised. One report, dealing with goats, suggested that a phenothiazine-salt mixture may have had deleterious effects when fed during the breeding season. The second stated that treatment with phenothiazine just before the breeding season could probably interfere with the ewes' settling to the service of the ram but that the phenothiazine-salt mixture does not interfere with conception. Behrens[1] states that rams should not be given phenothiazine during the mating season. Harwood,[3] in a comprehensive review of the anthelmintic properties of phenothiazine, makes no reference to its effect upon reproduction in sheep, except to state that it may cause abortion in late stages of pregnancy.

It has been demonstrated rather conclusively that continuous use of phenothiazine-salt mixtures is not detrimental to reproduction in sheep. Rietz and Wilson[5] used the medicated mixture for four years and observed no ill effects in breeding efficiency. Price, Hardy, and Boughton[4] found no difference in lamb production between control animals and an experimental group that received the medicated mixture for as long as seven years. Workers in the Animal Disease and Parasite Research Branch of the Agricultural Research Service,[6] reporting on the successful use of the medicated mixture for a ten-year period, recorded a satisfactory lamb crop.

The present experiment was undertaken to determine the effect, if any, of full therapeutic doses of phenothiazine administered to ewes one day before the start of the breeding season.

EXPERIMENTAL DATA

In the fall of 1954, about 500 aged, range ewes from three ranches in New Mexico were brought to State College for an artificial insemination experiment. The phenothiazine study reported here was set up within the framework of this major experiment. Thirty-two ewes selected at random from each ranch for the phenothiazine experiment made a total of 96 ewes for the study. Forty-eight ewes, 16 from each ranch, received 25 Gm. of phenothiazine (N.F. green, Lederle) in the form of a drench the day before breeding started. The experiment was planned so that half the ewes from each ranch would be bred naturally and half artificially. Balance in numbers was maintained as nearly equal as possible between method of breeding and treatment in each group of ewes. The ewes were fed high-quality alfalfa hay while they were on the experiment, and improvement in their condition during the course of the experiment was observed.

The ewes were checked daily for estrus. If a ewe was not observed in heat within three weeks after breeding, it was concluded that pregnancy had occurred, and the ewe was slaughtered shortly thereafter. Pregnancy was determined by an examination of the uterus. The embryos were approximately 25 days of age when pregnancy determinations were made. Ewes that did not conceive at the first service were rebred twice more, if necessary.

RESULTS AND DISCUSSION

Figure 1 shows the cumulative number of ewes in the treated and control groups which were found in estrus daily as the breeding season progressed. By the end of two estrous cycles (34 days), 88 ewes, 44 in

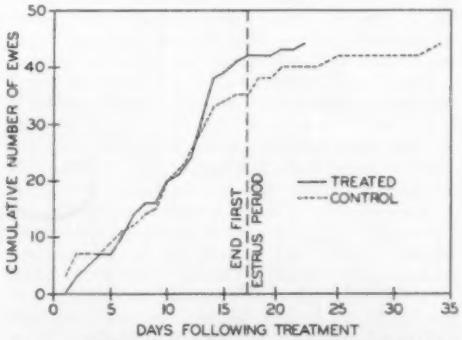

Fig. 1—Cumulative frequency distributions of first estrus during the breeding season.

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each group, had been observed in heat. As the graph shows, phenothiazine therapy had no detrimental effect on the occurrence of estrus in these ewes.

The effect of phenothiazine treatment on conception rate was determined from conceptions at first service in the 88 ewes which had exhibited estrus the first 34 days of the breeding season (table 1). Statistical analysis indicated that no significant differences existed between treatments, method of breeding, or origin of ewes, nor were there any interactions among these factors. Average conception rate for both the treated and control ewes was the same, 65.9 per cent. This is further evidence that the phenothiazine treatment just prior to the breeding season did not adversely affect this phase of reproduction.

TABLE 1—Conception Rate of Ewes Treated with Phenothiazine and of Untreated Ewes

	Treated	Controls
Number of ewes	48	48
Number observed in estrus*	44	44
Number conceived to first service	29	29
Conception rate (%)	65.9	65.9
Total pregnancies**	44	42
No. singles	41	39
No. twins	3	3

* During the first 34 days; ** includes conceptions from repeat breedings.

The 8 ewes that failed to show visible signs of estrus during the first thirty-four days of the breeding season were then placed in a pen with a fertile ram for six weeks. Three of the 4 treated ewes eventually conceived, and 2 of the 4 ewes in the control group conceived. One in the latter group carried a mummified fetus.

A total of 44 and 42 pregnancies, respectively, eventually occurred in the treated and control groups. There were three sets of twins in each group. The data on total pregnancies further substantiate the conclusion that phenothiazine has no adverse effect on conception in ewes that are treated just before breeding starts in the fall.

Since the ewes were not allowed to lamb, the findings of this study pertain only to the effect of phenothiazine on the early phases of reproduction, namely, estrus, conception, and early embryonic development. Further research is needed to determine its effect on the complete reproductive cycle.

SUMMARY

Results of an experiment on the effect of phenothiazine treatment on reproduction in ewes showed no detrimental effects on the occurrence of estrus, conception to first service, total pregnancies during the breeding season, or frequency of twinning. Forty-eight ewes were treated with phenothiazine, and an equal number served as controls.

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Operation for Otitis Externa in Dogs According to Zepp's Method

A Statistical Analysis of Follow-Up Examinations and a Study of Possible Age, Breed, or Sex Disposition to the Disease

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OTITIS EXTERNA is a common disease in dogs which is relatively resistant to medical treatment. Many cases in which medical treatment proves fruitless are, therefore, as a "last attempt" subjected to surgical treatment. It seldom happens that a case of otitis externa is primarily subjected to surgical operation.

From a prognostic point of view, the material presented must be considered fairly unfavorable. From a pathologico-anatomical viewpoint and certainly also etiologically, it is a heterogeneous material showing changes in the auditory duct in the form of ulcerations and chronic inflammatory processes of catarrhal-purulent or hypertrophic nature.

LITERATURE CITED

Different methods of operation have been developed for the surgical treatment of this disease, thus we have methods described by Hoffman,[6] Berge,[2] Formston and McCunn,[4] Srnetz,[16] Hinz,[6, 7] Schnelle[13] (Lacroix's method), Niemand,[11] and Zepp.[17, 18] These authors have worked out their respective, technically different methods but the fundamental principles are the same, namely, to bring about drainage and to make it easier to treat the auditory duct. An exception to this principle, to some extent, is Hoffman's method in which an opening is made ventrocaudally in the auditory duct for inserting a drainage tube. Berge and Srnetz split the auditory duct to such an extent that all ulcers, proliferations, et cetera, are layed bare for treatment. Drainage is brought about by tamponing but not suturing the incision. Formston and McCunn make an incision into the skin to expose the lateral wall in the vertical part of the auditory duct. Of the exposed part of the cartilage, a piece is resected in the form of an isosceles triangle with its point at the junction between the vertical and horizontal part of the auditory duct. Along the edges of the incision, the skin on the outside of the ear is sutured to the skin on the inside of the auricle and the auditory duct. Hinz splits the whole external auditory duct laterally and ventrally, respectively, as far as the meatus acusticus externus osseus. After that, a V-shaped piece of cartilage is resected, the apex of which reaches the beginning of the osseous part. The outer skin is then sutured to the skin inside the auditory duct. Niemand has modified Hinz's method by removing part of the subcutaneous fat and connective tissue from the bottom of the wound to reduce the strain on the sutures.

In Lacroix's method as described by Schnelle, two curved forceps or similar instruments are applied with one shank (jaw) on the inside of the vertical part of the auditory duct and the other on the skin on the outside of the ear with their convex borders facing each other and as wide apart as the space in the ear allows. Two incisions are then made through all the tissues along the convex borders of the forceps down to the level of the curve of the auditory duct. After removal of this V-shaped section of the ear, the outer skin is sutured to the skin inside the auricle and the auditory duct.

Zepp has modified the Formston and McCunn method which, in his opinion, has the following weak points: part of the parotid gland is included in the V-shaped portion of the tissue which is resected; the superficial temporal vein and the facial nerve are easily injured; a constriction of the auditory duct can easily occur through the formation of new granulation tissue and scar retraction. Also the hair that remains covering the orifice of the auditory duct becomes matted with the exudate formed in the auditory duct thus blocking the escape of the exudate. In order to eliminate these disadvantages, Zepp has worked out a method with a pedicle cartilage flap. A lateral triangular flap of skin is first resected, its base being at the auricle and its apex about 2 cm. below the point where the auditory duct curves in a horizontal direction. The incisions are then made into the lateral wall of the auditory duct, one at the front edge and one at the back edge, down to the point where the auditory duct curves horizontally. The upper part of the resected cartilage is cut off and a triangle, with its apex upward, is formed of the lower part which is folded ventrally and sutured to the skin in the V-shaped defect made by the skin resection. Dorsally the suturing is performed as before. The pedicle flap of cartilage prevents the auditory duct from being constricted by granulation tissue or scar retraction. Furthermore, almost all the hair is eliminated ventrally in the orifice of the auditory duct.

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METHODS

All surgical operations for otitis externa, in the Surgical Clinic of the Veterinary College of Stockholm, have been performed according to Zepp's method since Fritz Nilsson [12] introduced it after a study tour to the United States in 1947. According to experience gained here, the method gives better results than the one previously used, which corresponded closest to Lacroix's method.

With the exception of 30 cases in which Blakely [3] made follow-up examinations, the author has not been able to find any data on results obtained with Zepp's method. Therefore, all the cases at our clinic to Dec. 31, 1953, were studied and, whenever possible, were subjected to follow-up examinations. Necessary information was obtained by writing or telephoning the owners of the dogs. Some were willing to bring their dogs for a clinical examination. The form of the questions put to the owners was schematic. The owner had to decide only whether his dog had: (1) recovered completely; (2) had improved; or (3) had not improved after operation.

TABLE 1—Frequency of Otitis Externa in Different Breeds of Dogs

Breed	Distribution of breeds among licensed dogs in Stockholm, 1947–1953		Frequency of disease in different breeds in the "Stockholm material," 1947–1953		Significance
	No.	(%)	No.	(%)	
Spaniel	5,434	7.2	29	15.3	***
Alsatian	6,653	8.8	17	8.9	.....
Poodle	4,076	5.4	28	14.7	***
Fox Terrier	7,648	10.1	23	12.1	.....
Airedale Terrier	3,885	5.1	20	10.5	***
Schnauzer	2,423	3.2	10	5.3	.....
Boxer	5,558	7.4	10	5.3	.....
Mongrel	7,186	9.5	10	5.3	***
Harrier	1,283	1.7	5	2.6	.....
Scottish Terrier	2,118	2.8	6	3.2	.....
Spitz	3,538	4.7	2	1.1	*
Rottweiler	780	1.0	3	1.6	.....
Collie	4,313	5.7	3	1.6	*
Setter	990	1.3	1	0.5	.....
Elkhound	1,078	1.4	2	1.1	.....
Vorstehhund	464	0.6	2	1.1	.....
Dachshund	6,509	8.6	1	0.5	***
Sealyham Terrier	419	0.6	3	1.6	.....
English Bulldog	311	0.4	1	0.5	.....
Doberman Pinscher	449	0.6	1	0.5	.....
St. Bernard	92	0.1	1	0.5	.....
Newfoundland	286	0.4	1	0.5	.....
Chow Chow	269	0.4	2	1.1	.....
West Highland White Terrier	132	0.2	1	0.5	.....
Afghan Hound	170	0.2	2	1.1	.....
Brussels Griffon	215	0.3	1	0.5	.....
Pointer	250	0.3	1	0.5	.....
Iranian Shepherd	0	0.0	1	0.5	.....
Pekingese	1,907	2.5	1	0.5	.....
Irish Wolfhound	23	0.0	1	0.5	.....
Great Dane	184	0.2	1	0.5	.....
Others	6,800	9.0	....	....	.....
Total	75,443	99.7	190	100.0	.....

In determining the frequency of disease in different breeds and at different ages, the cases had to be divided into two groups, the Stockholm material and the rural material. The mother population consisting of licensed dogs * was known only for the Stockholm material.

RESULTS

From Sept. 1, 1947, to Dec. 31, 1953, 353 dogs were operated on for otitis externa according to the method introduced by Zepp. In 80.7 per cent of these animals, both ears were subjected to operation. The mortality was 1.7 per cent, and the median time for clinical treatment was 15.8 days—the lower quartile being 13.7 and the upper 19.0 days. In the 4 animals where necropsies could be made the causes of death were pneumothorax, acute hepatitis, hepatitis contagiosa canis, and cerebral hemorrhage, respectively. Two dogs were not subjected to necropsy.

In the follow-up examinations requested information was obtained on 281 (88.9%) of the animals. Of the 281 cases, 49.5 per cent had recovered completely, 15.6 per cent showed obvious improvement, and 34.9 per cent showed very little or no improvement. Of the dogs in the latter group, 37.8 per cent (13.2%) of the total were killed on account of the ear disease.

Many authors, among others, Georgi,[5] Krall,[9] Schulze,[14] Schuster,[15] and Srnetz [16] consider certain breeds, especially the ones that have drooping ears and whose auditory ducts are covered with much hair, predisposed to otitis externa. For this reason the present author investigated the breed incidence in his material. However, since there may also be an age disposition, the dogs were sorted also according to age. As material from a surgical clinic must be considered selected, it may be impossible to determine whether the disposition is a matter of breed, age, or both. If dogs at a certain age or of a certain breed should be predisposed to the disease, one could expect the condition in such an age category or such a breed to be more common, more severe, and the results of medical treatment to be less satisfactory and these cases over-represented in the surgical material. However, reduced healing should also be noticeable in the results of surgical treat-

* Dogs for which taxes, compulsory on all dogs in Sweden, have been paid to the city.

ment as no new factor is in principle brought into the course of healing. The only difference is that better ventilation is provided and the exudate can more easily be discharged. Reversely, one can say, therefore, that, if the results of surgical treatment should prove to be independent of the dog's breed as well as of its age, the same thing would hold good of medical therapy. Consequently, selection would be of no importance.

If the dogs which originated from the Stockholm material are now classified according to their respective breeds (table 1), it will be seen that certain breeds such as the Spaniel, Poodle, and Airedale Terrier are especially predisposed to the disease while Dachshunds, mongrels, and possibly Spitz dogs and Collies are more resistant.

In the same way an "age disposition" is found when the material is tested after classification according to the dogs' ages (table 2). As previously mentioned, a breed disposition might be a mere age effect in the event dogs of a susceptible age should appear in great numbers. In order to find out to what extent such conditions might be present, the author divided his material into five age groups (table 3). From this table it is seen that Airedale Terriers are amply represented at 5 to 8 years of age where the risks of the disease seem to be greater. On the other hand, the Spitz breed is represented by many dogs over 10 years old, where the risks are slighter. An explanation could be that the proved breed disposition would be the consequence of the age distribution of the breeds. But, on the other hand, if Spaniels and Dachshunds are compared to each other, it is seen that these breeds are fairly equally represented in the two age groups in question but very unequally from the viewpoint of disposition. One must, therefore, believe that an actual breed disposition very likely exists.

TABLE 2—Frequency of Otitis Externa in Dogs in Different Age Groups

Age (years)	Age distribution of licensed dogs in Stockholm, 1947–1953		Frequency of otitis in the "Stockholm material," 1947–1953		Relative frequency (2/1 · 1,000)
	No. 1	(%)	No. 2	(%)	
< 1	8,332	11.1	3	1.6	0.4
1 to 2	8,856	11.8	17	8.9	1.9
2 to 3	11,768	15.7	27	14.2	2.3
3 to 4	9,655	12.9	26	13.7	2.7
4 to 5	7,493	10.0	25	13.2	3.3
5 to 6	5,674	7.6	22	11.6	3.9
6 to 7	4,615	6.2	29	15.3	6.3
7 to 8	4,233	5.7	14	7.4	3.3
8 to 9	3,863	5.2	12	6.3	3.1
9 to 10	2,771	3.7	8	4.2	2.9
>10	7,557	10.1	7	3.7	0.9
Total	74,817	100.0	190	100.1	....

Result of χ^2 test of the values shown in this table was $\chi^2_{10} = 57.47.^{***}$

TABLE 3—Breed Distribution of Dogs of Mother Population Among Some Age Groups

Breed	<1 yr. (%)	1 to 5 yr. (%)	5 to 8 yr. (%)	8 to 10 yr. (%)	>10 yr. (%)
Spaniel	16.4	54.7	18.7	6.8	3.5
Poodle	16.1	54.6	16.1	7.3	5.8
Airedale Terrier	10.7	41.0	28.2	12.0	8.2
Mongrel	18.1	45.5	20.6	8.8	7.0
Spitz	15.3	40.7	20.5	9.2	14.3
Dachshund	17.0	54.1	18.8	6.6	3.5
Others	9.8	50.8	19.3	9.1	11.1
Total	11.1	50.5	19.4	8.8	10.1

Concerning the surgical treatment the question will then be: Does the dog's breed or age influence the result? Will the final result be better in a Dachshund or mongrel than in a Spaniel or Poodle?

In order to get an answer to these questions, the answers to the previously mentioned inquiries were grouped according to breed (table 4A) and according to age (table 5A). After that the different breeds were grouped together in the following way: 1—breeds predisposed to the disease; 2—more or less resistant breeds; 3—other breeds. A comparison was then made between all three breed groups as well as between groups 1 and 3 and between groups 2 and 3. In no case could a significant difference be found, nor did age prove to influence the results of treatment. As it might be possible that these results could be influenced by some owners not having answered our inquiries concerning their dogs, these dogs were also grouped and analyzed according to breed and age (tables 4B and 5B). It was then seen that the not-answered cases had no influence on the breed groups ($\chi^2_2 = 2.9$). The significance of the age factor was, on the other hand, $_4 = 10.0^{*}$) which might be due to the

TABLE 4A—The Result of Surgical Treatment in Different Breeds of Dogs Calculated in Percentage Recovered (A) and Improved (A)

Breed	A	B	Total	A (%)	Breed groups*	A	B	Total	A ave. (%)
Spaniel	31	15	46	67.4					
Poodle	22	9	31	71.0	1	70	32	102	68.6
Airedale Terrier	17	8	25	68.0					
Mongrel	10	2	12	83.3					
Spitz	3	4	7	42.9					
Collie	4	2	6	66.7	2	22	8	30	73.3
Dachshund	5	0	5	100.0					
Others	91	58	149	61.1	3	91	58	149	61.1
Total	183	98	281	.....	....	183	98	281	.....

A = Dogs perfectly well and also those which improved; B = *status quo*.
* 1 = Breeds predisposed to the disease; 2 = more or less resistant breeds; 3 = all other breeds.
$\chi^2_2 = 2.52$ (breed groups 1-2-3). $\chi^2_1 = 1.5$ (breed groups 1-3). $\chi^2_1 = 1.61$ (breed groups 2-3).

large percentage of "not-answered" cases in age group 5. The result of treatment was thus neither influenced by the breed of the dog nor by its age.

TABLE 4B—The Result of the Follow-Up Examination in Different Breed Groups of Dogs Calculated in Percentage of Answers Plus Untraceables

Breed groups	Answers plus untraceables	No answers	Total	Answers plus untraceables (%)
1	112	7	119	94.1
2	32	4	36	88.9
3	167	22	189	88.4
Subtotal	311	33	344	90.4
Unknown breed	0	2	2	.....
Total	311	35	346	.....

Result of χ^2 test of the values shown in this table was $\chi^2_2 = 2.90$.

Of the 281 dogs in which final results were determined, 12.8 per cent showed diminished hearing power.

DISCUSSION

From follow-up examinations of the surgical cases it is seen that 49.5 per cent of the dogs completely recovered, 15.6 per cent improved to a considerable extent, and 34.9 per cent showed no change or were only slightly improved. Keeping in mind that the cases dealt with are chronic, have resisted long medical treatment, and have twice as great a possibility of ending up in the group of *status quo*, since the disease in many cases appears in both ears, we must say that the results of the surgical treatment are satisfactory. However, about 35 per cent of our cases did resist surgical treatment also. Previously we believed that the poor results in such animals were due to otitis media which supported the otitis externa by its exudate. Examinations of some of these dogs by Månsson [10] indicates that this is not always the case. It has also been shown that dogs with a perforated tympanic membrane

TABLE 5A—The Result of Surgical Treatment in Different Ages of Dogs Calculated in Percentages of Dogs Perfectly Well and Those Which Improved

Age (years)	A	B	Total	A (%)	Age groups	Total	A (%)
0 to 1	3	1	4	75.0	1	4	75.0
1 to 2	15	8	23	65.2			
2 to 3	32	15	47	68.1			
3 to 4	36	13	49	73.5	2	157	66.2
4 to 5	21	17	38	55.3			
5 to 6	24	8	32	75.0			
6 to 7	21	15	36	58.3	3	94	63.8
7 to 8	15	11	26	57.7			
8 to 9	7	2	9	77.8			
9 to 10	5	4	9	55.6	4	18	66.7
> 10	4	4	8	50.0	5	8	50.0
Total	183	98	281	.....	....	281	.....

Results of χ^2 test on individual dogs 1 to 10 years old was $\chi^2_{10} = 8.02$. Results of χ^2 tests on dogs in five age groups was $\chi^2_4 = 0.93$.
A = Perfectly well and also those which improved; B = *status quo*.

have completely recovered. In order to diagnose the perforation, radiographs have been taken after injection of a contrast medium into the auditory duct of the dog while lying on its side, the cavum tympani being filled if a perforation exists. On the other hand, remaining trouble has been found in cases where the tympanic membrane has been intact.

Examinations of dogs, in which symptoms reappear after Zepp's operation, reveal grave changes in the form of ulcerations and constrictions in the remaining (horizontal) part of the external auditory duct. In 9 such animals the meatus acusticus externus cartilagineus has, therefore, been totally extirpated (Månsson). As a result of this operation, 6 of the 9 dogs are free from symptoms and perfectly well. One of the dogs is free from symptoms with the exception of a fistula at the site of the auditory duct and 2 which did not recover were killed. One of these dogs was examined in the clinic one month after surgery. At this time it did not show any symptoms of disease but had a fistula at the previously mentioned place.

One of the 9 dogs referred to showed symptoms of injury to the labyrinth. Upon arrival at the clinic, the dog could not hold its head straight and suffered from balance disturbances. In this case the meatus acusticus externus cartilagineus was primarily totally extirpated. After that, the symptoms quickly disappeared and the dog recovered completely. Another dog, first operated on for otitis media by means of trepanation and drainage of the bulla ossea with little benefit, also recovered after total extirpation of the meatus acusticus externus cartilagineus. The results of this method are consequently promising and the method may perhaps become a good complement of Zepp's operation. The results of the operations also seem to indicate that changes in the external auditory duct may be of importance in the occurrence of symptoms from the labyrinth. Therefore, continued investigations into this field seem justifiable.

Concerning the causes of the breed disposition, the present observations do not render definite conclusions possible. There are, however, no grounds for believing that the shape of the external ear is of any great importance since there is no uniformity in the shape of the ears either in predisposed or resistant breeds. Berg's [1] investigations concerning dogs with cropped ears indicate that this intervention was of no importance to the frequency of otitis externa. The importance of a hairy auditory duct is difficult to judge because of the great individual variations that are found in this respect in any one breed.

TABLE 5B—The Result of the Follow-Up Examination in Different Age Groups of Dogs Calculated in Percentage of Answers Plus Untraceables

Age groups	Answers plus untraceables	No answers	Total	Answers plus untraceables (%)
1	5	1	6	83.3
2	176	12	188	93.6
3	97	12	109	89.0
4	23	2	25	92.0
5	9	4	13	69.2
Subtotal	310	31	341	90.9
Unknown age	1	4	5	.....
Total	311	35	346	.....

A probable result of χ^2 test of the values shown in this table is $\chi^2_4 = 10.0$.*

That factors other than purely anatomical ones may play a part in the frequency of the disease is seen from the proved age disposition.

SUMMARY

The author has made a comparison and statistical analysis of 353 postexamined dogs subjected to Zepp's operation for otitis externa in the surgical clinic of the Veterinary College of Stockholm. Of these dogs, 80.7 per cent had been bilaterally operated. The median of the time for treatment in the clinic was 15.8 days. Mortality was 1.7 per cent, but 4 of the 6 deaths were apparently due to unrelated causes. Follow-up examinations were possible in 281 (88.9%) of the animals, with 49.5 per cent recovering completely, 15.6 per cent showing improvement, and 34.9 per cent showing little or no improvement. Of the latter group, 38.8 per cent were killed on account of the ear disease.

An analysis shows that certain breeds are predisposed to the disease. There is also a marked age disposition. Comparative calculations indicate that the breed disposition is not a mere age effect. There is no definite sex disposition.

The results of the surgical treatment

proved to be independent of the dog's breed or age. In 12.8 per cent of the cases, the owners considered the hearing of their dogs to be impaired. Of the 34.9 per cent not benefited by the operation, Månsson [10] found that this was due to changes in the remaining part of the auditory duct. Total extirpation of this part in 9 dogs resulted in 7 of them becoming free from symptoms and a fistula remained in 1.

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The Response of Newborn and Young Ferrets to Intranasal Administration with Egg-Adapted Distemper Virus

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CANINE distemper exists as an epizootic contagious disease in dog populations throughout the world. The extreme probability of exposure of young dogs, and the almost uniform high susceptibility of this age group, have indicated that vaccination, if it is to be effective, must be accomplished at an early age. Too often the commercial antiserum dosage commonly employed fails to protect the puppy against field exposure with virulent distemper virus (DV). The earliest age at which young dogs can be protected is not known. A similar situation prevails in young mink; however, in the face of outbreaks it is often necessary to vaccinate nursing kits. In order to provide some correlating evidence for early vaccination trials in dogs, foxes, and mink, it was thought desirable to test the response of newborn and young ferrets, from resistant and susceptible dams, to the administration of egg-adapted distemper virus.

MATERIALS AND METHODS

Strains of Virus.—The virus used for vaccine in these studies was living egg-adapted distemper virus of the eighty-eighth to ninetieth egg passage level of the Onderstepoort strain. Test virus was cultivated in seven-day embryonating eggs by chorioallantoic inoculation of 0.2 ml. of a 2×10^{-1} dilution of seed virus. After six days' incubation at 37 C., the chorioallantoic membranes were harvested and combined with equal quantities of inactivated (56 C.) horse serum. Grinding was accomplished in a Waring blendor. The suspension was then centrifuged at 2 C. in an international refrigerated centrifuge for thirty minutes at 6,000 r.p.m.; the supernatant fluid was removed, mixed, distributed in 2-ml. quantities and stored in a mechanical freezer at —20 C. until used. The virus pools were free from bacterial contamination.

For challenge purposes, the Green (distemperoid) strain of ferret passaged virus was employed. This strain has been maintained in this laboratory by transfer in ferrets. The spleen, liver, and lungs were removed from moribund infected ferrets and frozen at —20 C. At the time of challenge, the tissues were thawed and ground in a Waring blendor using sterile nutrient broth (Difco) to make a 20 per cent weight/volume suspension. One milliliter was administered by the intramuscular route for challenge.

Animals.—Black-Footed and albino ferrets of both sexes were housed in sheds and fed a ration consisting of horsemeat, liver, and fish (50 %) and commercial cereals (50 %). Immune dams were provided by vaccination with egg-adapted virus and challenged ten days later with Green's distemperoid virus. Both exposures were given in 1-ml. quantities by intramuscular injection approximately four to five months prior to whelping.

EXPERIMENTAL RESULTS

Newborn and young ferrets were vaccinated at varying intervals following parturition by giving each animal 1 drop of vaccine in each nostril. Table 1 summarizes the results obtained when the vaccinated progeny from immune and susceptible dams were challenged with virulent virus. It was found that there was a marked difference in the response to immunization. With the exception of the 35-day age group, young from susceptible dams were receptive to immunization on or after the eighth day of age. When the young from the immune dams were vaccinated and subsequently challenged, the data indicate that under the conditions of this experiment these young must be at least 36 days of age before they can be successfully immunized.

DISCUSSION

It would appear that naturally transmitted passive immunity from the ferret dam controls to some extent the response of the young to immunization. Direct correlation of these findings in ferrets to observations on the response of the newborn and young dogs is not yet possible. The present authors have carried out studies

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TABLE 1—Vaccination of Newborn and Young Ferrets

From unvaccinated dams			From vaccinated dams		
Age (days) at time of vaccination	Days between vaccination and challenge	Results*	Age (days) at time of vaccination	Days between vaccination and challenge	Results*
2	88	0/4	2	91	0/3
3	83	1/6	3	91	0/4
8	68	5/5	8	79	0/7
10	73	1/1	10	79	0/8
19	60	5/5	18	63	0/5
35	39	1/7	19	63	0/3
47	25	5/5	36	39	0/7
61	11	7/7	47	30	4/4
Unvaccinated controls		0/4	Unvaccinated controls		0/4

* Numerator—number of surviving ferrets; denominator—number challenged with virulent virus.

which indicated that progeny from hyperimmune dams respond to vaccination with egg-adapted virus at 7 weeks of age. Similarly, studies on the advisability of hyperimmunizing the bitch and early vaccination of weaned puppies are under way.

It must be pointed out that our experiment was not designed to test the duration of immunity within the group of young from vaccinated or unvaccinated dams but only to compare the ability of the young to respond to the immunizing agent. Although the young from the vaccinated dams were challenged at different intervals following exposure to the egg-adapted virus, these intervals were approximately the same as those in the corresponding groups from unvaccinated females. The effect of a possible interference phenomenon, maturation of antibody-forming mechanism, local active immunity, or low grade antibody response should be common to both groups.

SUMMARY

The response of vaccinated newborn and young ferrets from immune and susceptible dams to challenge with virulent distemper virus was studied. Young ferrets from dams susceptible to distemper were receptive to immunization at 8 days of age; whereas, young from immune dams were refractive to vaccination until thirty-six to forty-seven days after parturition.

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Successful Immunization of Mink with a Toxoid Against *Clostridium Botulinum*, Type C

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RAW MEAT and raw fish products constitute the major part of the modern diet of domesticated mink. In many instances, because of careless handling of these products ranchers have suffered severe financial losses from botulism toxemia, due to the contamination of food by *Clostridium botulinum*, type C. The disease is world-wide, but reports indicate that it has not been a serious problem for other domestic animals, except among cattle in South Africa which have a habit of feeding on carrion. It has become prevalent enough in domesticated mink to warrant generalized vaccination of these fur-bearing animals against botulism.

The objective of this study was the preparation of a successful toxoid from *Cl. botulinum*, type C, toxin for protection of mink and other animals which might be subjected to the hazards of type C botulism.

LITERATURE REVIEW

Quortrup and Gorham [1] found that mink were highly susceptible to *Cl. botulinum*, type C, toxin and moderately susceptible to types A and B toxin. Moberg and Kull [2] confirmed these findings with studies of numerous outbreaks in mink in Sweden, where botulism was positively identified as the cause of 13 of 22 epizooties of food poisoning and type C toxin was proved responsible for 12 of the 13. One outbreak was not identified as to type, due to lack of toxin-containing food materials.

In the experience of two of the authors (AEL and PSN), two epizooties of food poisoning among mink have been studied, though not reported, and in both cases, the agent was positively identified as type C botulinum toxin.

Two major problems associated with the preparation of *Cl. botulinum*, type C, toxoid are: (1) the inability of a given strain of the type C organism to continue to synthesize toxin under laboratory conditions and (2) the lability of the toxin itself. It is generally conceded that the preparation of toxoid is dependent upon the production of toxin of high potency and high antitoxin-combining power. The only information to the contrary was that of Boroff and Cabeen [3] who described the immunization of rabbits parenterally with washed cells of an atoxic variant of *Cl. botulinum*, type C. These rabbits not only produced agglutinating and flocculating antibodies but were protected against challenge with at least 20,000 mouse minimum lethal dose (m.l.d.). These researchers were also able to show passive protection of mice with pooled serums from the immunized rabbits against 1,000 mouse m.l.d. of *Cl. botulinum*, type C, toxin.

Stevenson, Helson, and Reed [4, 5] described a simplified medium of a corn steep liquor base which supported an excellent growth of *Cl. botulinum*, types A and B, with large and consistent yields of toxin.

Poulson and Sterne [6] were able to produce high yields of botulinum toxin, types A and B, by growing the organisms in physiological NaCl solution contained in cellophane bags immersed in meat broth. The L_f and L_+ doses* were increased about eight times that of cultures grown by ordinary means. They demonstrated that if the cellophane bags were filled with broth, instead of salt solution, the *Cl. botulinum* organisms produced very little toxin. The loss in antigenicity during the preparation of toxoid as measured by the flocculation test was about half that found with toxoids produced in meat broth culture.

MATERIALS AND METHODS

Cultures.—Clostridium botulinum, type C, cultures were obtained from two sources, the Rocky Mountain Laboratory, U. S. Department of Health, Education, and Welfare, Hamilton, Mont., and K. E. Kull, of the State Veterinary Medical Institute, Stockholm, Sweden.

The Rocky Mountain Laboratory sent two cultures, one in the lyophilized form and one in liquid culture. In all tests in our laboratory, the lyophilized culture showed the highest production of toxin, and maintained the better stability of the two. Cultures were maintained in the laboratory in two different mediums. Thioglycolate broth and a corn steep liquor-powdered milk-glucose medium of Lewis and Hill.[7]

Medium and Apparatus.—The medium utilized for the laboratory production of toxin was a modification of that described by Sterne and Wentzel.[8, 9]

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* L_f—the amount of toxin which produces optimal flocculation with 1 unit of antitoxin; L_+—a toxin-antitoxin mixture which contains one fatal dose in excess and which will kill the experimental animal.

The medium as finally constituted was prepared as follows:

	Per Liter
Corn steep liquor (crude)	300 ml.
Casein	5 Gm.
Calcium chloride	1 Gm.
Tap water (8.5) to make	1,000 ml.

pH adjusted to 7.5

A further modification of the medium from that of Sterne and Wentzel[8,9] was to disregard the precipitate which would form in the medium upon sterilization. Where steps were not taken to remove precipitable solids before the preparation of the final medium, sterilization in the autoclave caused a heavy precipitate to form. When utilizing the cellophane bag technique, however, there was no inhibition of toxin formation, even when there was a copious amount of precipitate surrounding the bags in which the cultures were growing.

The culture medium after preparation was placed in a 20-liter pyrex 12 by 12 inch jar over which a stainless steel lid was fitted. The lid contained five holes which would accept No. 3 rubber stoppers. A short length of 8.0-mm. glass tubing was placed through each stopper and an 11-inch length of cellophane sausage casing (2¼ in. dia.) was firmly attached to each glass tube. The distal ends of the casings were closed by dampening and knotting each several times and tying each with string both above and below the knots. Fifteen liters of medium were placed in the jar around the cellophane sacks, and 1 liter of isotonic NaCl solution placed in each of four of the sacks. The jar and its contents were sterilized at 15 lb. of pressure for three hours, allowed to cool and the saline of each sack was then inoculated with *Cl. botulinum*, type C Swedish strain, which had been prepared in corn steep liquor-powdered milk-glucose broth. The cultures were incubated at 37 C. for four days and the toxin, which was in the cellophane sacks, was harvested and tested for potency.

Preparation of the Toxoid.—The toxin was treated with formalin to a final concentration of 0.5 per cent (0.19% formaldehyde) and adjusted to pH 5.8 by the addition of hydrochloric acid. The preparation was incubated at a temperature of 37 C. for ten days to complete detoxification of the material and then tested for toxicity. No attempt was made to separate the *Cl. botulinum*, type C, spores and vegetative cells from the toxin. According to Boroff and Cabeen[3] these would add to the immunogenicity of the preparation. One tenth volume of 10 per cent potassium alum was added and the mixture allowed to stand at room temperature overnight. This resulted in precipitation of the toxoid, from which the supernatant was siphoned and replaced with an equal volume of sterile isotonic sodium chloride solution. Finally the suspension was adjusted to pH 6.8 with sodium hydroxide. This constituted the toxoid which was used in the immunization procedures described.

Toxin Potency Tests.—Four groups of 12 mice each were injected intraperitoneally with 0.025, 0.0025, 0.00025, and 0.000025 ml. of toxin, respectively, and observed for ninety-six hours.

Toxoid Toxicity Tests.—Guinea pigs in two groups of 4 each were injected subcutaneously with 1 ml. or 5 ml. of the formalin-treated toxin, respectively, and observed for ninety-six hours. Also 4 mink were each injected subcutaneously with 5 ml. of toxoid. All animals were repeatedly examined throughout the observation time for any signs of generalized toxemia or of local inflammation.

Immunization of Mink.—Mink which were soon to be killed for their pelts were chosen for the immunization tests. The 76 chosen were divided into five groups and treated as follows.

In group 1, 12 animals were given two subcutaneous injections of 1 ml. of toxoid, fourteen days apart, and were challenged with active toxin fifty-three days after the last injection.

In group 2, 12 animals were injected as were the first group except that immunization was begun fourteen lays later. These animals were challenged with active toxin thirty-seven days after the last toxoid injection.

In group 3, 20 animals were injected with a single, subcutaneous 1-ml. dose of the toxoid and were challenged thirty-eight days later.

In group 4, 12 animals were each injected with a single intramuscular 1-ml. dose of the toxoid and challenged twenty-seven days later.

In group 5, 20 nonimmunized control mink were fed active toxin at the time the immunized animals were challenged.

Challenge.—One hundred milliliter of toxin, prepared by the method described, was mixed with 10 lb. of regular mink feed. This toxin when assayed killed all mice injected intraperitoneally in concentrations of 0.0025 ml. within seventy-two hours (table 1). To ensure consumption of the toxin-containing ration, the mink were fasted for twenty-four hours prior to feeding. Each animal received approximately one fourth pound of food containing an estimated 2.5 ml. of toxin, about 10,000 mouse m.l.d. per feeding. The challenge ration under the conditions of the fast was eagerly and quickly consumed.

Because of the high potency of the toxin, and the possibility that all the animals would die when challenged, not all were challenged on the same day. Some were held in reserve to be fed smaller amounts of toxin in case the initial challenge dose was overwhelming. Consequently, the test mink were given one, two, or three feedings of the toxic ration.

RESULTS AND DISCUSSION

Toxin Potency Tests.—Table 1 presents the results of toxin potency tests. All mice receiving 0.00025 ml. of more of toxin died

within twelve hours. A dose of 0.000025 ml. of toxin killed within ninety-six hours only 4 animals of 12 injected.

Toxoid Toxicity Tests.—None of the 8 guinea pigs or 4 mink given 1 ml. or 5 ml.

TABLE 1—Results of Potency Tests of Toxin

No. of mice	Dose intraperitoneal (ml.)	Total deaths	Within hours
12	0.025	12	6
12	0.0025	12	12
12	0.00025	12	72
12	0.000025	4	96

of toxoid, as described, showed local or general symptoms of toxemia.

Immunization Tests.—The results of the experiment were decisive. None of the 56 vaccinated mink showed any signs of botulism, and all of the 20 control animals died within twenty-four hours from the time of the first toxin feeding except 2. Of these 2, one did not eat the second time, but both died within twelve hours of the second feeding (table 2).

TABLE 2—Results of Mink Immunization Tests

Group	No. of mink	Toxoid injections: Route	No.	Days before challenge	Mortality after challenge
1	12	SC*	2	68 and 53	0/8**
		SC	2	67 and 53	0/4
2	12	SC	2	53 and 37	0/12
3	20	SC	1	38	0/20
4	12	IM†	1	37	0/6
		IM	1	36	0/6
5	20	Control	0	....	20/20
Cumulative Results		Immunized			0/56
		Nonimmunized			20/20

* SC—subcutaneous; † IM—intramuscular.
** The numerator, the number of deaths; the denominator, the number challenged.

In ordinary field outbreaks, death is usually much slower than shown by the mink of this control group. The animals may linger two or three days before dying, and some show no signs of poisoning for several days after exposure, then they become acutely ill and die. This would indicate that the immunized animals were protected against much larger amounts of toxin than they would probably receive in naturally contaminated food under field conditions.

There was no indication that one route of administration of the toxoid has an advantage over another. Also the data show that one injection of toxoid is as effective as two for the production of complete immunity to the usual accidental exposure to *Cl. botulinum*, type C, toxin in contaminated food.

Further studies are underway to learn the duration of protection to be expected from a single immunizing dose of toxoid. Also, quantitative serological studies of the serums from immunized animals are in progress and will be the subject of another paper in the near future. Also, the storage qualities of the toxoid are under investigation, both under ideal and less favorable storage conditions.

Summary

1) Toxin of *Clostridium botulinum*, type C, was prepared by growing the organism in physiological sodium chloride solution contained in cellophane bags which were immersed in a corn steep liquor medium. High yields of toxin were obtained by this method.

2) Formolized, alum-precipitated toxoid was prepared from toxin and utilized to immunize 56 mink which were marked for pelts.

3) Challenge of the immunized and of the control mink showed decisively that the immunized animals were completely protected against challenge doses of toxin which killed all control animals within thirty-six hours.

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PH Values and Enzymatic Activity of the Digestive Tract of the Chicken

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THE PH of various regions of the digestive tract in the chicken has been determined by a number of investigators. A review of the literature reveals a considerable amount of variation in their data.

Buckner and his associates [4] report a pH of 4.4 in the proventriculus while Heller and Penquite [13] state that the pH of this region is 3.61. Mussehl *et al.* [18] found the average pH of the upper small intestine to be 6.2, but Farner [9] reports a maximum pH of 5.90 in this area. Olson and Mann [19] report that pH values appearing in the cecums vary from 5.90 to 8.20 with a mean of 7.12, but Farner [9] reports an average pH of 5.71 in the cecums. The fact that previous reports showed a considerable amount of variation in the pH of the digestive tract of the chicken prompted this investigation.

Sturkie [22] states that relatively little is known about digestion in birds and practically no work has been conducted in recent years. A review of the literature supports this statement and reveals the lack of knowledge of the enzymatic activity of the digestive tract of the chicken. Also, the presence of enzymes in certain regions of the alimentary canal has been a subject of controversy. Plimmer and Rosedale [20] report the finding of carbohydrate and protein-splitting enzymes in crop tissue or crop mucosa, but Shaw [21] found no enzymes present in extracts of the crop mucosa.

There is also a question as to carbohydrate digestion in the gizzard and no work has been recorded in regard to the presence of lipolytic enzymes in the gizzard. Farner [9] and Sturkie [22] report the presence of amylolytic enzymes in bile. Plimmer and Rosedale [20] determined the presence of enzyme activity in certain regions in a neutral medium except for the small intestine where he studied the enzymatic activity in acid and alkaline mediums. In view of the fact that there is little or no reported data on the enzymatic activity in the chicken at different pH levels, an experiment was designed to determine the proteolytic, lipolytic, and amylolytic activity at different levels of pH in extracts of various regions of the digestive tract in the chicken.

MATERIALS AND PROCEDURE

Ration.—Thirty White Leghorn cockerels, 4 months of age, were used in this experiment. They were maintained on a ration, for the first three months, composed of ground yellow corn, wheat middlings, meat and bone meal, dehydrated alfalfa meal, soybean oil meal, condensed fish solubles, D-activated animal sterol (source of vitamin D_3), niacin, calcium pantothenate, choline chloride, riboflavin supplement, vitamin B_{12}, antibiotic feeding supplement, cod liver oil, vitamin A feeding oil and minerals (salt, manganese sulfate, calcium carbonate, and bone meal). The ration carried a guaranteed analysis of crude protein, not less than 20 per cent; crude fat, not less than 3 per cent; crude fiber, not more than 4.5 per cent; and minerals, 3 per cent. Thirty days prior to the experiment, the ration was modified by withdrawing the condensed fish solubles and vitamin A feeding oil and by adding iron oxide, ground oats, and wheat bran. The guaranteed analysis of this ration was crude protein, not less than 19 per cent; crude fat, not less than 3 per cent; crude fiber, not more than 8 per cent; and minerals, 4 per cent. The cockerels were allowed the ration and water *ad libitum*.

PH Determinations.—The chickens were killed by rapid bleeding and the samples were removed immediately. The bile was collected from the gall bladder and the pancreatic tissue was finely divided by means of a scissors and ground with sand in a mortar. Samples from the crop, proventriculus, gizzard, intestine, and cecums were prepared by suspending the material from the lumen in three volumes of distilled water. The bile and ground pancreas were also diluted similarly. Previous tests by Farner [9] revealed that the digestive contents were sufficiently buffered to withstand dilutions of 1:5 without altering the pH of the material.

The coarse material was filtered, and the pH of the filtrate was determined with a Beckman model G pH meter. The apparatus was standardized daily with a standard buffer solution.

Preparation of Enzyme Solutions.—In 15 cockerels, the enzymes of the digestive tract were extracted with glycerol and in the remaining specimens the extractions were made with distilled water. The samples were obtained immediately after removal of the material for the determination of the pH of the various regions.

The gizzard lining and pancreas were cut into small pieces and ground with sand in a mortar. The ground mass of the first 15 chickens was put into glycerol and the material from the last 15 was put into distilled water. After twenty-four hours of refrigeration, the supernatant liquid was strained through cheese cloth to remove sand and large pieces.

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The proventriculus, a glandular organ, yielded a yellowish, viscous secretion when pressed with a scalpel. The secretion was then mixed with glycerol or distilled water and used for the testing of enzyme activity.

The mucous membranes of the remaining parts of the alimentary canal were scraped with a scalpel and put into glycerol or distilled water. No doubt the scrapings included some material of the digestive tract in addition to a possible secretion of the mucosa.

Enzyme activity tests were conducted by dividing 48 cc. of each extract into eight equal parts, placing 6 cc. of each in eight beakers. To each beaker were added 24 cc. of solutions consisting of definite hydrogen ion concentrations which were prepared and adjusted to the following pH levels: 1.2, 2.5, 3.6, 4.9, 5.7, 7.0, 8.5, and 10.0, respectively. The contents of each of the eight beakers were divided into six equal parts and placed in test tubes which gave six tubes of diluted extract at each of the pH values, or forty-eight tubes in all.

Carbohydrate Hydrolysis.—Amylolytic activity was demonstrated by incubating the diluted extract with a starch solution and utilizing Benedict's test to determine the presence of activity. The carbohydrate digestion tests were made by taking tubes number 1 and 2 from each of the eight pH values. One of these tubes from each pH value was used as a control and no starch was added. To tube 2 of each pH value, 1 cc. of 10 per cent starch solution was added. All of the tubes were placed in an incubator for twenty-four hours at 37.5 C. and tested for reducing substances by boiling not more than 8 drops of the solution with 5 cc. of Benedict's solution for two minutes.[12]

Protein Hydrolysis.—A test to determine the presence of proteolytic enzymes was carried out on egg albumin. The egg albumin was separated from the yolk and diluted with a sufficient amount of 0.85 per cent NaCl to make a 10 per cent solution. Tubes 3 and 4 from each of the eight different pH values were used for the test. Tube 3 from each of the pH values was used as a control and 1 cc. of 10 per cent egg albumin was added to tube 4 of each pH value. All of these test tubes were placed in an incubator at 37.5 C. for twenty-four hours and then tested with the biuret test for the presence of proteoses and peptones.[12]

Fat Hydrolysis.—The lipase activity was tested with wesson oil by using phenolphthalein, bromthymol blue, methyl red, methyl orange, and thymol blue as indicators. Of the remaining sixteen tubes, tube 5 from each pH value was used as a control and 2 cc. of wesson oil was added to tube 6 of each pH value. The tubes were incubated for twenty-four hours at 37.5 C. and observed for lipolytic activity by a change in indicator color due to the production of fatty acids.

TABLE 1—The pH Mean and Range of the Digestive Tract of 30 Chickens

Region	Mean pH	Range pH	pH
Crop	4.67	4.39	4.82
Proventriculus	4.48	4.30	4.60
Gizzard	2.94	2.83	3.01
Duodenum	6.13	5.95	6.40
Jejunum	6.29	6.03	6.62
Ileum	6.58	6.36	6.81
Cecums	6.14	5.75	6.50
Rectum	6.82	6.62	7.21
Bile (gall bladder)	6.51	6.12	6.64
Pancreas	6.58	6.38	6.80

Results and Discussion

PH of the Alimentary Canal.—The range and mean of the pH values in bile, pancreatic tissue, and various areas of the alimentary canal of 30 chickens are presented (table 1).

The lowest pH values in the digestive tract were observed in the gizzard. This supports Friedman's[10] observations that the secretion of the proventriculus was very acid and that this secretion is collected in the gizzard for digesion purposes. Farner[6] points out that the pH of the proventriculus is higher than the gizzard because material obtained from the proventriculus is apparently a mixture of an acid

TABLE 2—Proteolytic, Amylolytic, and Lipolytic Activity in Various Regions of the Digestive Tract of 30 Chickens at Different pH Levels

	Carbohydrate								Protein								Fat							
pH levels	1.2	2.5	3.6	4.9	5.7	7.0	8.5	10.0	1.2	2.5	3.6	4.9	5.7	7.0	8.5	10.0	1.2	2.5	3.6	4.9	5.7	7.0	8.5	10.0
Crop	—	—	—	sl	xx	xxx	x	—	x	xx	x	—	—	—	—	—	—	—	—	—	x	x	x	—
Proventriculus	—	—	—	—	—	—	—	—	xx	xxx	xx	sl	—	—	—	—	—	—	—	—	—	—	—	—
Gizzard	—	—	—	sl	x	xx	x	—	xx	xxx	xx	sl	—	—	—	—	—	—	—	—	x	x	x	—
Duodenum	—	—	—	sl	x	xxx	x	—	x	xx	x	x	x	xxx	x	—	—	—	—	—	x	xx	x	—
Jejunum	—	—	—	x	xx	xxx	xx	sl	x	xx	x	x	x	xxx	x	—	—	—	—	—	xx	xxx	x	—
Ileum	—	—	—	sl	x	xx	x	—	x	x	x	—	x	x	x	—	—	—	—	—	x	xx	x	—
Cecums	—	—	—	—	x	x	x	—	x	x	x	—	sl	x	x	—	—	—	—	—	x	xx	x	—
Bile	—	—	—	—	x	x	x	sl	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Pancreas tissue	—	—	—	sl	x	xxx	x	sl	—	—	—	—	x	xx	x	—	—	—	—	—	xx	xxx	x	—

xxx High degree of enzymatic activity; xx moderate degree of enzymatic activity; x small degree of enzyme activity; sl slight enzyme activity; — no observable enzymatic activity.

serous secretion and a more alkaline mucous secretion.

All specimens revealed a gradual increase in pH values from the duodenum through the rectum excluding the cecums. The rectum exhibited the highest pH of the digestive tract and the only region to present an alkaline pH. This was observed in only 2 chickens. The mean pH of the cecums was 6.14 which is about the same as the duodenum but lower than the jejunum, ileum, and rectum.

The average pH of bile was found to be 6.51 with a range from pH 6.12 to 6.64. This is considerably higher than the pH mean of bile reported by Mayhew [16] but nearly equal to 6.49 which was reported by Heller and Penquite.[13] Extracts of pancreas showed an average pH of 6.58 and exhibited a pH range of 6.38 to 6.80.

Enzymatic Activity.—Little or no difference appeared in the results of the enzyme activity which might be due to a difference in the extractive materials. However, there was some evidence (table 2) that the enzyme extractions made with distilled water did not possess as high a degree of lipolytic activity as the glycerol extractions.

Digestion in the Crop.—Amylolytic, proteolytic, and lipolytic enzymes were present in scrapings of the crop mucosa (table 2). The carbohydrate-splitting enzymes were active over a pH range of 4.9 to 8.5 with the greatest amount of activity appearing at a pH of 7.0. The proteolytic enzymes present appear to have been regurgitated from the proventriculus since they were active at a pH range of 1.2 to 3.6. It must be kept in mind that the average pH of the crop as reported previously in this paper was 4.67 and the crop enzymes did not show optimum activity at this pH value. While the pH present in the crop does not allow maximum enzyme activity, one may surmise that some protein and carbohydrate digestion is performed in this region. Since the lipase present did not reveal any activity at a pH of 4.9 or below, there is reason to doubt any lipolytic activity in the crop.

Digestion in the Proventriculus and Gizzard.—In view of the data compiled (table 2) the only major enzyme present in the secretion of the proventriculus is pepsin. The proteolytic activity was present from a pH range of 1.2 to 3.6 with some evidence of the presence of proteoses and peptones at a pH of 4.9. Since it was found that the average pH of the proventriculus and gizzard was 4.48 and 2.94, respectively, it would appear that the gizzard was the site of peptic activity. There also was evidence of carbohydrate and fat-splitting enzymes in the gizzard. However, since there was no lipase activity at a pH of 4.9 or below and no amylolytic activity at pH 3.6 or below, there seems sufficient evidence to believe that only proteolytic activity is present in the gizzard.

Digestion in the Small Intestine and Cecums.—The largest amount of enzymatic activity was exhibited in the intestine. Pepsin and trypsin-like enzymes appeared present since proteolytic activity was observed over a pH range of 1.2 to 8.5 with a maximum amount of activity appearing at a pH of 2.5 and 7.0. It is also observed (table 2) that the proteolytic activity decreased in the terminal part of the small intestine with no protein splitting present at a pH of 4.9 in the ileum.

Carbohydrate-splitting enzymes were quite active in the small intestine over a pH range of 4.9 to 8.5 with a decrease in activity in the ileum. There was a slight amount of amylolytic activity observed in the jejunum at a pH of 10.0, and in the duodenum and ileum at pH 4.9. There is reason to believe that the major portion of the carbohydrate digestion is accomplished in the intestine since our observations (table 2) reveal little amylolytic activity in other regions of the alimentary canal at the pH of those regions.

The lipase activity appeared quite constant throughout the intestine with a somewhat greater amount of activity present in the jejunum. The lipolytic activity appeared over a pH range of 5.7 to 8.5 with the largest amount of fat hydrolysis at a pH of 7.0.

Carbohydrate, protein, and fat-splitting enzymes were found in the scrapings from the mucosa of the cecums (table 2). It is highly possible that these enzymes were carried into the cecums with the intestinal mass. Plimmer and Rosedale [20] report the absence of any enzymes in the mucosa of the cecums.

Enzymes Present in Bile and Pancreas Tissue.—Carbohydrate-splitting enzymes were the only digestive enzymes present in the bile. They were inactivated at a pH of 4.9 or below and exhibited slight activity

at pH 10.0. They did not present as high a degree of amylolytic activity as the carbohydrate-splitting enzymes of the small intestine or pancreas.

Proteolytic, amylolytic, and lipolytic activity was present in the pancreatic tissue. The protein-splitting enzymes revealed the greatest amount of activity at a pH of 7.0 and were active from pH 5.7 to 8.5. Carbohydrate-splitting enzymes were slightly active at a pH of 4.9 and 10.0 but exhibited a considerable amount of activity within this range. Lipase revealed its greatest activity at a pH of 7.0 and was active over a pH range of 5.7 to 8.5.

Summary

1) The lowest pH value was observed in the gizzard.

2) All specimens revealed a gradual increase in pH from the duodenum through the rectum, excluding the cecums. The rectum exhibited the highest pH of the digestive tract and the only region to present an alkaline pH which was observed in only two specimens.

3) Unlike the alkaline pH (7.8) of bile in mammals, the mean pH of the bile from the chicken was 6.51.

4) While the pH of the crop does not allow maximum enzyme activity, some protein and carbohydrate digestion is performed in this region. There appears to be no lipolytic activity in the crop.

5) The only major enzyme present in the secretion of the proventriculus is apparently pepsin. The average pH of the proventriculus and gizzard is 4.48 and 2.94, respectively; therefore, it would apear that the gizzard was the site of peptic activity.

6) Carbohydrate and fat-splitting enzymes were present in the gizzard but presented no activity at the pH in this region.

7) The largest amount of enzymatic activity was exhibited in the intestines.

8) Since little amylolytic activity prevailed in other regions of the alimentary canal, the major portion of the carbohydrate digestion is apparently accomplished in the intestines.

9) Carbohydrate-splitting enzymes were present in the bile.

10) Proteolytic, amylolytic, and lipolytic activity was observed in extracts of the pancreatic tissue and presented activity at a pH present in the intestine.

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Xanthomatosis in Chickens

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THE PURPOSE of this paper is to describe an uncommon condition affecting the skin and wattles of chickens.

HISTORY

It is not known whether the history of this flock had any causal relationship to the skin condition. The flock was comprised of 800 White Leghorn chicks from strain A and 800 White Leghorn chicks from strain B. Strain A birds were identified by amputation of the comb. Sulfaquinoxaline was fed continuously in the mash, at the rate of 0.0125 per cent, as a coccidiostat, until the birds were moved to range at 3 months of age. The sulfaquinoxaline was then discontinued and nitrosal* was fed in the mash for three months at the rate of 2 lb. per ton of mash. When the birds were about 4 months of age, a weed killer containing 2-4-D and 2-4-5-T was sprayed on a nearby hedgerow in which a part of the birds usually roosted.

At 6 months of age, the flock became sick and the owner instituted treatment consisting of 1 tablespoonful of muriate of potash in each gallon of drinking water plus 30 Gm. of penicillin per ton of mash. As the birds did not respond, sulfaquinoxaline was added to the water for the next forty-eight hours.

Twelve birds died and 5 were submitted for necropsy, revealing hemorrhages in the spleen, nephritis, ascites, and hydropericardium. One bird had a mottled liver and large, irregular necrotic areas in the spleen. Bacteriological examination was negative. Medication was discontinued and mortality stopped.

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Thanks are due to Dr. M. Kare for cholesterol determinations, and to Dr. J. Bentinck-Smith for the clinical studies. Appreciation is expressed to Drs. P. Olafson and P. P. Levine for their advice and assistance. The author is also indebted to Dr. C. A. Gleiser, Armed Forces Institute of Pathology, Washington, D. C., for the histological study and photomicrographs.

* Trade name for Dr. Salsbury's Laboratories. Contains 30 per cent N^4-Acetyl-N^1-(4-nitrophenyl) sulfanilamide and 9 per cent 3-Nitro-4-Hydroxyphenylarsonic acid.

Fig. 1—Chickens with xanthomatosis—normal bird (left); affected bird (right). Note swollen wattles and thickened skin on legs, abdomen, and breast of bird on right.

Fig. 2—Chicken with xanthomatosis. Note swellings on lateral aspect of tibia and dimpled effect caused by depression of feather follicles.

Two weeks later, as the pullets were being placed in two separate laying houses, they were individually handled and examined and nothing unusual was observed.

Six weeks later, it was noticed that about 5 per cent of the pullets had swollen wattles. They were in good production and there was no mortality. Younger and older groups of birds in adjoining pens were unaffected.

Clinical and Laboratory Findings

In December, 1952, five 8-month-old birds were submitted to our laboratory. Some of the birds had unilateral or bilateral swelling of the wattles (fig. 1) containing a gelatinous honey-colored transudate. The skin on the breast and abdomen was thickened and doughy and contained a transudate similar to that seen in the wattle.

The birds were bright, alert, and in production. Examination of the internal organs revealed nothing unusual. Bacteriological examination of the wattles, skin, and internal organs was negative.

Several months later, additional specimens were submitted. About 40 per cent of the flock was now affected, mostly from strain A since only about 2 per cent of the birds of strain B were affected. The character of the skin lesion had changed from being soft and doughy and had become quite firm. The thickness of the skin lesion, from the surface epithelium to the underlying muscle, was about 1.25 cm. The epidermis was an abnormal orange-yellow and there was no longer a gelatinous honey-colored transudate in the subcutis. The tissue beneath the epidermis was taffy-colored and firm. Chalky-white areas were interspersed throughout abnormal subcutaneous tissues. Thickened areas of skin were also found on the lateral and medial aspect of the tibia. In time the affected areas on the legs enlarged. Some became spherical in shape and attained a diameter of 2.5 to 7.5 cm. (fig. 2), with marked dimpling where the feather follicles were deeply indented (fig. 2 and 3). Some birds had a cystic swelling on the breast up to 7.5 cm. in diameter which contained a yellow, watery fluid.

Histological examination of the affected skin and wattles showed a xanthomatous-type lesion, consisting of foam cells, fibroblasts, lipoid droplets, and lenticular spaces (fig. 4, 5, and 6). Frozen, unstained sections when viewed with a polarizing microscope, revealed birefringent, rhombic crystals and the typical maltese cross effect of cholesterol esters.

The Schultze's histochemical test for cholesterol or related substances[9] was positive on frozen sections taken from the skin lesions. Cholesterol determination of the pathological skin and subcutaneous tissue by the method of Abell *et al.*[1] showed a content of 4,282 mg./100 Gm. of abnormal skin as compared to 154 mg./100 Gm. of normal skin. Cholesterol determination of fluid in the skin cysts showed 476 mg./100 ml. of fluid.

Fig. 3—Chickens with xanthomatosis—normal bird (left); affected bird (right). Note nodular swellings in skin on medial aspect of tibia and thickened masses of skin in axilla of bird on right.

Attempts to correlate these skin lesions with abnormal variations in the blood components were unsuccessful. Total blood cholesterol, as determined by the method of Bloor *et al.*,[5] was well within the normal limits. The alkaline phosphatase of the serum was normal. The van den Bergh tests, both direct and indirect, were negative.

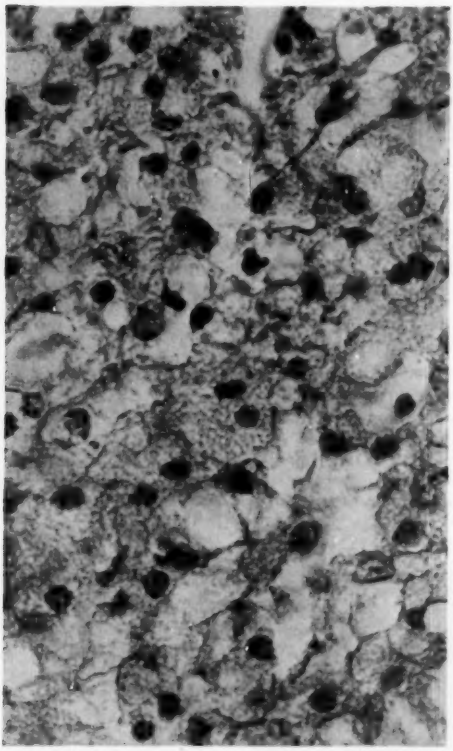

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Fig. 4—Histological section of affected skin of chicken with xanthomatosis showing the foamy nature of the cytoplasm in the foam cells. x 1,000.

ETIOLOGY

Attempts to find the etiological agent of this condition have failed. Since the abnormal birds had been exposed to a weed killer containing 2-4-D and 2-4-5-T, it was decided to see what effect this material would have when applied to the skin of normal birds. Six White Leghorn pullets were painted on various areas of the body for eight consecutive days with weed killer and observed for six months. Noticeable changes did not develop in these birds.

Injection of cell suspensions from affected tissues intradermally, subcutaneously, and intramuscularly did not transmit the condition.

Numerous reports of xanthomatosis in man, following administration of organic arsenicals,[2, 9, 10] prompted us to try these compounds on chickens. The flock, in which this condition appeared, had been fed 2 lb. of nitrosal per ton of mash for three months; therefore, 12 chickens, 6 weeks of age, were given 51 gr. of 3-Nitro-4-Hydroxyphenylarsonic acid per liter of drinking water for ten weeks. This caused no visible changes in the next six months.

DISCUSSION

There is a paucity of reports of xanthomatosis in domestic animals and birds. Nieberle [11] mentioned a xanthoma in a parrot but gave no description of the case. Jarmai [7] reported a generalized case in a horse. Nitsche [8] reported a localized lesion in a horse. In each of these cases the etiology was undetermined. Hudson [6] saw this condition in three flocks of White Leghorn chickens. In these cases, the incidence of

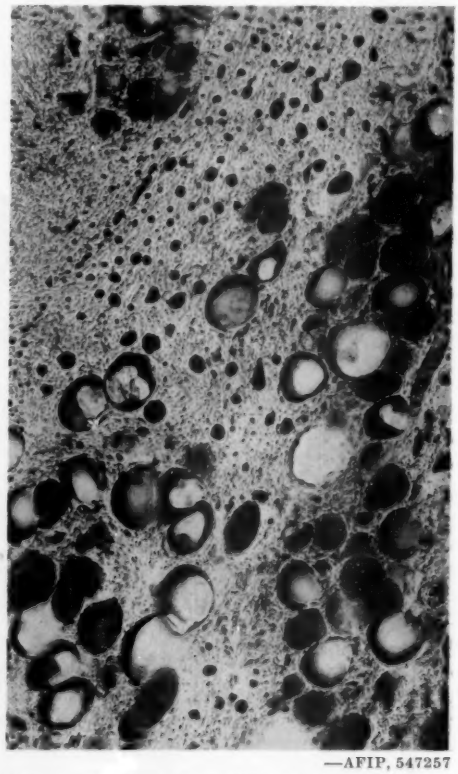

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Fig. 5—Histological section of the skin of a chicken with xanthomatosis stained with oil red "O" showing a large number of lipoid droplets in the subcutaneous connective tissue. x 160.

swollen wattles ranged from 1 to 5 per cent. In addition, some of the birds also had thickened yellow areas of skin. These birds were 6½ months to 9 months of age.

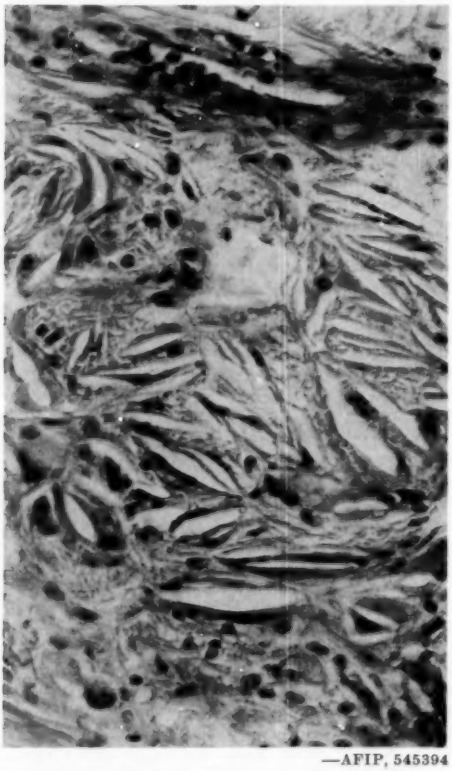

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Fig. 6—Histological section of affected skin of chicken with xanthomatosis showing lenticular spaces left by crystals dissolved in the dehydration process. x 470.

Nothing unusual had been done in the way of feeding or management in these flocks.

Boyd [4] described at least three forms of xanthomatosis in man. One form, seen primarily in elderly people, is characterized by yellow elevations on the eyelids. A second form, xanthoma multiplex, manifested by the presence of yellow nodules scattered over the body, is associated with high levels of cholesterol in the blood, and occurs in diabetes and obstructive jaundice. The third type occurs in the region of the tendon sheaths and may resemble giant-cell tumors. The condition we have described in chickens does not seem to resemble the forms described in man.

Summary

A case of xanthomatosis in a flock of chickens has been reported. Attempts to demonstrate an etiological agent were unsuccessful. This condition could not be correlated with metabolic dysfunction.

Reference is made to other cases of xanthomatosis in chickens and domestic animals. The counterpart of this condition was not found in reports of xanthomatosis in man.

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Hemolytic Activity of Newcastle Disease Virus

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LITERATURE CITED

The first report of a viral hemolysin was that associated with mumps virus and was by Morgan *et al.*[9] Shortly afterward, Kilham[7] described hemolysis caused by Newcastle disease virus (NDV). These two viruses are the only animal viruses known to lyse red blood cells (r.b.c.). This distinction naturally poses some interesting questions especially since these viruses are closely related biologically.

From the examination of the literature and from our initial attempts to obtain hemolysis of r.b.c. with mumps and Newcastle disease viruses, it was obvious that to effect strong hemolysis would require more than just mixing r.b.c. and freshly harvested, infective allantoic fluid. Morgan *et al.*[9] used isotonic citrate as a vehicle and obtained increased hemolysis with mumps virus while Morgan[10] obtained similar results with infective allantoic fluid that had been dialyzed against phosphate-buffered saline. Granoff and Henle[6] reported that freezing and thawing greatly enhanced the hemolytic potential of NDV.

These reports made it evident that factors and conditions involved in hemolysis as well as the hemolytic property itself should be re-examined. Also, since most reports concerning hemolysis by NDV usually have dealt with only one strain of the virus, it appeared logical that a comparative study of the hemolytic capacities of several strains of NDV should be made with the idea that the degree of hemolysis could be correlated with other known properties of the virus. The experiments which are described in this report were performed for the purpose of further elucidating these questions.

MATERIALS AND METHODS

Viral Strains.—The repository for strains of NDV is maintained in the Virus Laboratory, Department of Veterinary Science, University of Wisconsin, Madison, with the cooperation of the Agricultural Research Service, U. S. Department of Agriculture and the Newcastle Disease Technical Committee of the North Central States Region. From time to time, strains from various sources have been submitted to the repository until there are at present more than 80 strains, including 1 from Australia, 8 from Canada, 2 from Mexico, 1 from Japan, 8 from Europe, and the remainder from the United States. All repository strains were included in these investigations. For most of the developmental work, however, seven strains of NDV were used: Vic—Australia; Bl; Hert—Eng.—1933; Milano—Italy—1945; GB—Texas—1948; (H) Najarian—Ohio—1948; and Roakin—NJ—1946; also employed were the Enders strain of mumps virus and the Lee strain of Influenza B virus.

Eggs.—White Leghorn eggs from a commercial hatchery were employed. As far as could be determined, these eggs came from flocks not vaccinated or infected with Newcastle disease. Incubation preliminary to inoculation was in a forced-air, Jamesway incubator (model 252, single stage) at 98–99 F. The wet bulb thermometer readings ranged from 94 to 95 F.

Viral Pools.—Mumps Virus.—Eight-day embryonating chicken eggs were inoculated intra-allantoically with 0.05 ml. of a 10^{-2} dilution of stock allantoic fluid virus. Further incubation was at 98–99 F., all eggs being candled daily. After five days, the eggs with living embryos were removed from the incubator and chilled at 5 C. overnight. The allantoic fluids were harvested and pooled. If the fluids were to be used within four to five days, they were stored at 4 C.; otherwise, they were stored at —20 C.

Newcastle Disease Virus.—Chicken embryos, 10 or 11 days old, were inoculated intra-allantoically with 0.05 ml. of a 10^{-2} dilution of stock allantoic fluid virus. Inoculated eggs were incubated at 98 to 99 F. and candled two to three times daily. From embryos that died after twenty-four hours, allantoic fluids were harvested, pooled, and stored as described for mumps virus.

Lee Strain of Influenza B Virus.—Embryonating chicken eggs, 10 to 11 days old, were inoculated intra-allantoically with 0.05 ml. of a 10^{-2} dilution of stock allantoic fluid virus. After further incubation at 98 to 99 F. for sixty-five hours, the living embryos were chilled overnight and the allantoic fluids were harvested and handled as described previously for the other viruses.

Hemagglutination Tests.—Starting with a 1:5 dilution of allantoic fluid virus, serial twofold di-

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lutions were made in 0.25-ml. volumes of physiological saline. Chicken r.b.c., 0.25 ml. of a 1.0 per cent suspension in physiological saline, were thoroughly mixed with each dilution. The test was read after thirty to forty-five minutes at room temperature (26 C.). The end point (1 hemagglutination unit) was considered as the highest dilution of virus showing 1+ agglutination (button with slightly diffused margin). One plus agglutination was not evident in most instances as the last tube to show agglutination was usually 4+ (complete agglutination). Consequently, most 1+ values were obtained by interpolation. For example, if the highest dilution of virus showing agglutination was the 1:320 dilution and the degree of agglutination was 4+, the 1+ value was 320 plus three-fourths the difference between 320 and the next dilution, 640. This resulted in a value of 560 for 1+ agglutination.

Hemolytic Test.—Serial twofold dilutions of allantoic fluid virus were made in 1.0-ml. volumes of 2.0 per cent citrate-saline solution or in physiological saline solution, unless otherwise specified. The dilutions were held in a 37 C. water bath until the temperature was equalized. Five-tenths ml. of a 2.0 per cent suspension of fowl r.b.c., preheated to 37 C., was added to each dilution and thoroughly mixed. After incubation at 37 C. for twelve to fourteen hours, the degree of hemolysis was estimated visually. Complete hemolysis was recorded as 4+, 75 per cent hemolysis as 3+, 50 per cent hemolysis as 2+, 25 per cent hemolysis at 1+, a slight trace as t, and no hemolysis as negative.

Hemolytic-Inhibition Test.—Starting with a 1:5 dilution, serial two-fold dilutions of serum were made in 1.0-ml. volumes of physiological saline. One milliliter of dialyzed allantoic fluid, which had been frozen and thawed, was thoroughly mixed with each dilution. These mixtures were incubated at 37 C. for ten minutes after which 0.5 ml. of 2.0 per cent suspension of chicken erythrocytes preheated to 37 C. was added to each dilution. The degree of hemolysis was ascertained after fifteen hours at 37 C.

Dialysis of Allantoic Fluid.—Allantoic fluid to be dialyzed was placed in a section of 28-mm. cellophane dialyzing tubing which had been closed at one end by knotting. As soon as the fluid was placed in the tube, its open end was closed by another knot. The bags of allantoic fluid were placed in a large volume of physiological saline buffered at pH 6.6 with 0.001 M phosphate buffer. Occasionally, the bags and saline were agitated. Dialysis was allowed to proceed for thirty-six hours with the buffered saline being changed every twelve hours.

TABLE 1—Hemolytic Activity of Normal and Viral-Infected Allantoic Fluid at 37 C.

Allantoic fluid preparation	Dilution* of allantoic fluid					HA titer
	0	2	4	8	16	
Vic	t**	t	t	t	—	480
Hert–E	1	1	1	1	—	520
Milano	t	t	t	t	—	1280
GB	t	t	—	—	—	1920
Roakin	t	t	t	t	—	960
Naj	—	—	—	—	—	1920
Bl	t	t	—	—	—	1920
Mumps	—	—	—	—	—	80
Lee B	—	—	—	—	—	320
Normal	—	—	—	—	—	0

* In saline containing 2.0 per cent sodium citrate; ** —, t, 1 = 0, trace, and 25 per cent hemolysis, respectively; HA—hemagglutination.

Freezing and Thawing Allantoic Fluid.—Allantoic fluid preparations, contained in lusteroid tubes, were placed in a dry ice-ethanol bath and frozen quickly. As soon as freezing had occurred, the tubes were transferred to a 56 C. water bath to thaw. The freezing and thawing process was repeated four times.

Antiviral Serums.—Antiserums were prepared from 8- to 12-week-old chickens that had had no prior experience with NDV. Of the nonlethal strains of NDV, the chickens received one 0.5-ml. injection of undiluted, infective allantoic fluid, intravenously, and were exsanguinated for serum collection three weeks later. Of the more pathogenic strains of NDV, the initial injection was 0.5 ml. of formolized (0.1 %) allantoic fluid. This was followed some ten days later by injection of

TABLE 2—The Influence of Freezing and Thawing on Hemolytic Activity of Normal and Viral-Infected Allantoic Fluid

Allantoic fluid preparation	Dilution* of allantoic fluid										HA titer
	0	2	4	8	16	32	64	128	256	512	
Vic	t**	t	t	—	—	—	—	—	—	—	640
Hert–E	1	1	1	1	t	t	—	—	—	—	1120
Milano	1	1	1	1	1	—	—	—	—	—	1920
GB	1	1	1	1	1	t	t	—	—	—	1920
Roakin	1	1	1	1	1	1	t	t	—	—	1920
Naj	1	1	1	1	1	t	—	—	—	—	1280
Bl	1	1	1	1	1	1	1	1	1	—	5120
Mumps	—	—	—	—	—	—	—	—	—	—	80
Lee B	—	—	—	—	—	—	—	—	—	—	320
Normal	—	—	—	—	—	—	—	—	—	—	0

* In saline containing 2.0 per cent sodium citrate; ** —, t, 1 = 0, trace, and 25 per cent hemolysis, respectively; HA—hemagglutination.

0.5 ml. of 10^{-2} to 10^{-4} dilution of infective allantoic fluid, intravenously. Antimumps serum was prepared by giving four 2-ml. injections, intravenously, at five-day intervals and bleeding ten days after the last injection.

TABLE 3—The Influence of Dialysis on the Hemolytic Activity of Normal and Viral-Infected Allantoic Fluid

Allantoic fluid preparation	Dilution* of allantoic fluid					HA titer
	0	2	4	8	16	
Vic	1**	—	—	—	—	520
Hert–E	1	1	1	t	—	640
Milano	1	t	—	—	—	1920
GB	t	—	—	—	—	3840
Roakin	t	—	—	—	—	1920
Naj	t	—	—	—	—	960
Bl	2	t	—	—	—	2560
Mumps	—	—	—	—	—	240
Lee B	—	—	—	—	—	320
Normal	—	—	—	—	—	0

* In saline containing 2.0 per cent sodium citrate; ** —, t, 1, 2 = 0, trace, 25, and 50 per cent hemolysis, respectively; HA—hemagglutination.

Pretreatment of Red Blood Cells With Virus.—The source of r.b.c. was pooled blood collected from several adult White Leghorn and New Hampshire chickens in excess 2.0 per cent sodium citrate solution. Equal volumes of 2.0 per cent chicken r.b.c. suspended in calcium borate buffered saline (pH 6.8) containing 0.1 per cent calcium chloride and undiluted, allantoic fluid virus were mixed and incubated at 37 C. with occasional shaking. Three to four hours were usually required for the cells to become stabilized (not spontaneously agglutinable). After three to four hours these mixtures were centrifuged and the supernatant discarded. The sedimented cells were resuspended in citrate saline and centrifuged with the supernatant again being discarded. This process was repeated once. After the final washing, the cells were made up to a 2.0 per cent suspension in 2.0 per cent citrate saline solution.

Results

Effect of Dialysis and Freezing and Thawing on the Hemolytic Activity of Allantoic Fluid Virus.—A series of experiments consisted of testing dilutions of the following allantoic fluid preparations in 2.0 per cent citrate saline: (1) undialyzed (table 1), (2) undialyzed but frozen and thawed (table 2), (3) dialyzed (table 3), (4) dialyzed but frozen and thawed (table 4).

Hemolysis was most active in dialyzed preparations which had been frozen and thawed. The other three preparations were only slightly hemolytic. Neither the Lee strain of influenza virus nor normal allantoic fluid preparations caused hemolysis. The Enders strain of mumps virus was hemolytic. The hemagglutination titer was not altered appreciably by any of the treatments. All of the seven strains of NDV studied most extensively caused hemolysis, the Hert-Eng, Milano, Najarian, and Bl strains exerting greater activity than the Vic, GB, or Roakin strains. The remainder of the 81 strains of NDV also hemolyzed chicken r.b.c. but there was considerable variation in the degree among them. In view of these findings, subsequent studies were made only with allantoic fluid preparations which had been dialyzed and frozen and thawed.

Effect of Diluent on Hemolytic Activity of NDV.—Physiological saline, 2.0 per cent sodium citrate in physiological saline, and 0.1 per cent calcium chloride in physiological saline were studied (table 5). Saline and citrate saline gave excellent results, the former being slightly superior. Hemolytic activity was observed with calcium-saline as the vehicle but the extent was greatly reduced as compared to citrate-saline or saline.

TABLE 4—The Influence of Freezing and Thawing on Hemolytic Activity of Normal and Viral-Infected Dialyzed Allantoic Fluid

Allantoic fluid preparation	Dilution* of allantoic fluid											HA titer
	0	2	4	8	16	32	64	128	256	512	1024	
Vic	3**	3	3	2	2	2	1	t	t	t	—	640
Hert–E	4	4	3	3	3	3	2	1	1	1	—	960
Milano	4	4	4	4	3	3	2	2	1	t	—	1280
GB	4	3	3	3	3	3	2	2	1	t	—	1920
Roakin	3	3	3	3	3	3	2	1	1	1	—	1280
Naj	4	4	4	3	3	2	2	1	1	t	—	960
Bl	4	4	4	4	4	4	3	3	3	1	1	2560
Mumps	1	1	1	t	t	t	—	—	—	—	—	100
Lee B	—	—	—	—	—	—	—	—	—	—	—	320
Normal	—	—	—	—	—	—	—	—	—	—	—	0

* In saline containing 2.0 per cent sodium citrate; ** —, t, 1, 2, 3, 4 = 0, trace, 25, 50, 75, and 100 per cent hemolysis, respectively; HA—hemagglutination.

TABLE 5—The Influence of Various Diluents on the Hemolytic Activity of Newcastle Disease Virus-Infected Dialyzed Allantoic Fluids

Virus strain	Dilution of allantoic fluid											HA titer
	0	2	4	8	16	32	64	128	256	512	1024	
					DILUENT—SALINE							
Vic	3*	2	2	2	2	2	t	t	t	—		280
Hert–E	4	4	4	4	4	3	1	1	t	t	—	320
Milano	4	4	4	3	3	3	1	1	1	t	t	1920
GB	4	4	4	4	4	3	2	2	2	2	t	2240
Bl	4	4	4	4	4	3	2	2	2	1	—	1120
					DILUENT—2.0 PER CENT SODIUM CITRATE IN SALINE							
Vic	1	1	1	1	t	t	—	—	—	—	—	.....
Hert–E	3	3	3	3	2	2	1	t	t	t	—	.....
Milano	4	4	4	3	3	3	2	2	2	2	t	.....
GB	4	4	4	3	3	2	1	1	t	t	t	.....
Bl	4	4	3	3	2	2	2	1	1	1	—	.....
					DILUENT—0.1 PER CENT $CaCl_2$ IN SALINE							
Vic	....	1	1	1	1	t	—	—	—	—	—	.....
Hert–E	....	3	2	1	t	t	t	t	—	—	—	.....
Milano	....	3	3	2	1	1	t	t	t	t	t	.....
GB	....	3	2	1	t	t	t	—	—	—	—	.....
Bl	....	4	4	3	2	t	t	—	—	—	—	.....

* —, t, 1, 2, 3, 4 = 0, trace, 25, 50, 75, and 100 per cent hemolysis, respectively; HA—hemagglutination.

Effect of Temperature of Incubation on Hemolytic Activity of NDV.—The hemolytic activity of five NDV strains, Vic, Hert-Eng, Milano, GB, and Bl with incubation at 37 C., 25 C., and 5 C. are compiled (table 6). At 37 C. the degree of hemolysis was far greater than at 25 C. No hemolysis occurred at 5 C. The Hert-Eng strain failed to hemolyze at 25 C.

Effect of Storage at 5 C. and —20 C. on Hemolytic Activity of NDV.—The effect of storage on three strain of virus, Milano, GB, and Roakin, for periods up to forty-five days is recorded (tables 7 and 8). The hemolytic activity of NDV was but slightly reduced by forty-five days of storage. Possibly the reduction was slightly less at 5 C. that at —20 C.

Effect of 0.1 Per Cent Formalin on Hemolytic Activity of NDV.—The hemolytic activity of the Milano, GB, and Roakin strains was determined at one, three, sixteen, twenty-eight, and forty-five days after formolization (table 9). The hemolytic activity gradually disappeared during the course of the test period. After twenty-eight days, Milano strain exerted slight hemolysis, while Roakin and GB strains were inert. After forty-five days, the Milano strain lacked hemolytic activ-

TABLE 6—The Effect of Temperature of Incubation on the Hemolytic Activity of Newcastle Disease Virus

Virus strain	Dilution* of allantoic fluid										
	2	4	8	16	32	64	128	256	512	1024	2048
					37 C.						
Vic	3**	3	2	2	2	2	1	t	t	—	—
Hert–E	4	4	3	3	3	3	2	1	1	1	—
Milano	4	4	4	4	3	3	2	2	1	t	—
GB	4	3	3	3	3	3	3	2	1	t	—
Bl	4	4	4	4	4	4	3	3	3	1	1
					25 C.						
Vic	1	t	t	t	t	—	—	—	—	—	—
Hert–E	—	—	—	—	—	—	—	—	—	—	—
Milano	2	2	2	2	2	2	t	t	—	—	—
GB	3	3	2	2	1	t	—	—	—	—	—
Bl	2	2	2	1	1	1	1	1	—	—	—
					5 C.						
Vic Hert–E Milano GB Bl	No hemolysis										

* In saline containing 2.0 per cent sodium citrate; ** —, t, 1, 2, 3, 4 = 0, trace, 25, 50, 75, and 100 per cent hemolysis, respectively.

TABLE 7—The Effect of Storage for Various Periods at 5 C. on the Hemolytic Activity of Newcastle Disease Virus

Virus strain	Dilution* of allantoic fluid											HA titer
	0	2	4	8	16	32	64	128	256	512	1024	
						0 DAYS						
Milano	3**	3	3	2	2	2	1	t	t	—	—	1920
GB	4	3	3	3	3	3	2	2	1	t	—	3840
Roakin	3	3	2	3	3	3	3	1	1	1	—	1920
						AFTER 7 DAYS						
Milano	4	4	4	4	3	3	2	1	t	t	t	.....
GB	4	4	4	3	3	3	3	2	1	1	t	.....
Roakin	4	4	4	3	3	3	2	2	1	—	—	.....
						AFTER 14 DAYS						
Milano	4	4	3	3	3	3	2	1	1	t	—	.....
GB	3	3	3	3	3	3	2	1	1	t	—	.....
Roakin	3	3	3	3	3	3	2	t	t	—	—	.....
						AFTER 28 DAYS						
Milano	4	4	4	3	3	3	2	1	t	—	—	520
GB	4	4	4	3	3	3	2	2	t	t	—	1120
Roakin	3	3	2	2	2	2	1	t	—	—	—	1120
						AFTER 42 DAYS						
Milano	4	4	4	3	2	2	1	1	1	—	—	520
GB	4	4	4	4	2	2	1	1	1	t	—	1120
Roakin	2	2	2	2	2	1	1	1	—	—	—	640

* In saline containing 2.0 per cent sodium citrate; ** —, t, 1, 2, 3, 4 = 0, trace, 25, 50, 75, and 100 per cent hemolysis, respectively; HA—hemagglutination.

ity. The hemagglutination titer was reduced only slightly, if at all, after forty-five days.

Effect of Heating at 56 C. on Hemolytic Activity of NDV.—The results with Milano, GB, and Roakin strains are recorded (table 10). After five minutes at 56 C. only the Milano strain caused hemolysis and its titer was much less than that of unheated preparations. After ten minutes, Milano did not cause hemolysis. The hemagglutinin of the Milano and GB preparations was retained after ten minutes but in a greatly reduced strength.

Effect of Pretreating Red Blood Cells with Virus on Susceptibility to Hemolysis.—Burnet [2] reported that cells whose receptors had been removed by the receptor-destroying enzyme of *Vibrio cholerae* or by viral action resisted hemolysis. This prompted us to pretreat r.b.c. with virus and to test the susceptibility of the treated cells to hemolysis by strains of the same and another virus. The main objective was

TABLE 8—Effect of Storage at —20 C. on the Hemolytic Activity of Newcastle Disease Virus

Virus strain	Dilution* of allantoic fluid											HA titer
	0	2	4	8	16	32	64	128	256	512	1024	
						0 DAYS						
Milano	3**	4	4	4	4	3	2	2	2	1	t	1920
GB	4	4	4	4	3	3	2	2	2	2	t	1120
Roakin	2	2	2	2	2	2	2	2	t	t	t	1920
						AFTER 3 DAYS						
Milano	3	3	3	3	3	2	1	1	t	—	—	.....
GB	3	3	3	3	3	3	1	1	1	t	—	.....
Roakin	3	3	3	3	3	3	2	2	1	t	t	.....
						AFTER 16 DAYS						
Milano	4	4	4	4	3	2	1	t	—	—	—	1120
GB	3	3	3	3	3	3	2	1	t	—	—	520
Roakin	4	3	3	3	3	3	2	1	t	—	—	960
						AFTER 28 DAYS						
Milano	3	3	3	3	3	2	1	1	t	—	—	1920
GB	3	3	3	3	3	2	1	1	1	1	—	1920
Roakin	3	2	2	2	2	2	1	1	t	—	—	1920
						AFTER 45 DAYS						
Milano	4	4	4	2	2	1	1	1	—	—	—	1920
GB	3	3	3	2	2	2	1	1	t	—	—	1920
Roakin	2	2	1	1	1	1	t	—	—	—	—	1920

* In saline containing 2.0 per cent sodium citrate; ** —, t, 1, 2, 3, 4 = 0, trace, 25, 50, 75, and 100 per cent hemolysis, respectively; HA—hemagglutination.

to ascertain whether the action of one strain would prevent hemolysis by other strains. Seven strains of NDV, Vic, Hert-Eng, Milano, GB, Roakin, Najarian, and Bl and the Enders strains of mumps virus were employed in 2 trials (table 11). In the first trial, r.b.c. were in contact with virus for three hours and in the second trial the exposure time was extended to four hours. Data from the first trial suggested that the pretreatment exposure time was inadequate. After three hours, the action of Vic, Roakin, and mumps viruses prevented hemolysis by homologous virus, while Hert-Eng, Milano, GB, Najarian, and Bl virus-treated cells were still subject to partial hemolysis by homologous virus. When the pretreatment period was extended to four hours in trial 2, the r.b.c. previously treated with virus could not be hemolyzed by homologous viral strains. However, the Vic strain partially hemolyzed r.b.c. that had been pretreated with mumps virus. Hert-Eng and Milano viruses partially hemolyzed r.b.c. previously treated with Roakin, Najarian, Bl, and mumps viruses. The GB virus partially hemolyzed cells pretreated with Roakin and mumps viruses, and Najarian and Bl viruses partially hemolyzed r.b.c. pretreated with Roakin and mumps viruses.

Effect of Normal and Immune Serum on the Hemolytic Activity of Mumps and ND Viruses.—The results of using normal serum and antiserums to mumps, Roakin, and Calif 11914 (NDV) viruses are recorded (table 12). Antimumps serum completely prevented hemolysis by mumps virus but had no appreciable effect in inhibiting hemolysis by the ND viruses. Anti-NDV serum completely prevented hemolysis by NDV strains but were without effect on the hemolytic activity of mumps virus. Normal serum did not impair hemolysis by any of the viruses.

Discussion

Several types of interactions between NDV and r.b.c. have been postulated. Burnet and Lind [9] have theorized as follows. In type 1, there is a standard process of adsorption, enzymic action, and release of virus with fullest possible modification of the receptor. In type 1a, there are incomplete phases of the standard process, such as heating virus to 55 C., in which the virus is adsorbed firmly enough to allow visible agglutination but the enzymic action is restricted. Cells which have undergone the 1a type interaction are spontaneously agglutinable and small amounts of specific antiserum stabilize such cells by removing

TABLE 9—Effect of 0.1 Per Cent Formalin on the Hemolytic Activity of Newcastle Disease Virus

Virus strain	Dilution* of infected allantoic fluid											HA titer
	0	2	4	8	16	32	64	128	256	512	1024	
UNFORMOLIZED												
Milano	4**	4	4	4	4	3	2	2	2	1	t	1920
GB	4	4	4	4	3	3	1	1	1	1	t	1120
Roakin	2	2	2	2	2	2	2	2	t	t	t	1920
AFTER 24 HOURS												
Milano	4	3	3	3	3	2	1	1	t	—	—	.....
GB	4	4	4	3	3	3	2	1	1	t	—	.....
Roakin	4	4	4	3	3	3	2	1	t	—	—	.....
AFTER 72 HOURS												
Milano	3	3	3	3	3	3	1	t	—	—	—	.....
GB	3	3	3	3	3	3	2	1	1	—	—	.....
Roakin	4	4	4	4	3	3	2	1	t	t	—	.....
AFTER 16 DAYS												
Milano	2	2	2	2	2	2	t	t	—	—	—	1120
GB	2	2	2	2	2	2	1	t	—	—	—	1120
Roakin	2	2	2	2	2	2	t	—	—	—	—	520
AFTER 28 DAYS												
Milano	1	1	1	1	1	1	—	—	—	—	—	1920
GB	—	—	—	—	—	—	—	—	—	—	—	1280
Roakin	—	—	—	—	—	—	—	—	—	—	—	520
AFTER 45 DAYS												
Milano												520
GB	No hemolysis											1120
Roakin												1120

* In saline containing 2.0 per cent sodium citrate; **—, t, 1, 2, 3, 4 = 0, trace, 25, 50, 75, and 100 per cent hemolysis, respectively; HA—hemagglutination.

TABLE 10—Effect of Heating at 56 C. for Various Periods on the Hemolytic Activity of Newcastle Disease Virus

Virus strain	Dilution* of allantoic fluid									HA titer
	0	2	4	8	16	32	64	128	256	512	
0 TIME											
Milano	4**	4	4	4	3	2	2	1	1	—	1920
GB	4	4	3	3	3	2	1	1	1	—	3840
Roakin	3	3	2	2	2	2	1	1	t	—	960
5 MINUTES											
Milano	2	2	2	1	1	1	—	—	—	—	480
GB	—	—	—	—	—	—	—	—	—	—	520
Roakin	—	—	—	—	—	—	—	—	—	—	0
10 MINUTES											
Milano	No hemolysis										120
GB											480
Roakin											0

* In saline containing 2.0 per cent sodium citrate; ** —, t, 1, 2, 3, 4 = 0, trace, 25, 50, 75, and 100 per cent hemolysis, respectively; HA—hemagglutination.

the virus. In type 2, there is an irreversible union of virus particles to cell receptors. This occurs only above 28 C. and the attached virus particles can not be removed by receptor-destroying enzyme or immune serum. All the foregoing reactions can be demonstrated in the absence of hemolysis. This caused Burnet and Lind [9] to postulate another type of virus-receptor interaction (type 3) to account for hemolysis. Thus, in a virus population of infective allantoic fluid, there apparently exists a certain degree of heterogeneity with regard to adsorptive and enzymic activity. Burnet and Lind [9] term these viruses "variants" and imagine them as having different degrees of susceptibility to the influences of certain factors. For instance, according to the data presented here, the type 3 variant which causes hemolysis, is much more susceptible to inactivation by formalin, by heat, and by storage than is type 1 and possibly type 1a and 2 variants.

The effect of various chemical and physical factors is difficult to explain in most cases. Just why undialyzed preparations or dialyzed preparations, not frozen and thawed, are only weakly hemolytic, regardless of the diluent, is puzzling. It is known that allantoic fluid contains substances which inhibit hemolysis by these viruses. The cation, calcium, has been implicated as one factor. Morgan [10] believes that removal of calcium is the main result of dialysis. That this is not the whole story is evidenced by the fact that dialyzed preparations are hemolytic even though very weakly so, yet, on freezing and thawing,

TABLE 11—Effect of Pretreatment with Mumps and Newcastle Disease Virus on Hemolysis of Red Blood Cells by These Viruses

Virus	Red blood cells pretreated with various viruses								Hemolytic control*
	Vic	Hert–E	Milano	GB	Roakin	Naj	Bl	Mumps	
FIRST TRIAL (PRETREATMENT—3 HOURS AT 37 C.)									
Vic	—**	—	—	—	—	—	1	1	3
Hert–E	—	t	t	1	1	2	2	2	4
Milano	t	t	t	1	1	2	2	2	4
GB	t	—	—	1	1	1	2	2	4
Roakin	—	—	—	—	—	—	—	—	2
Naj	—	—	—	—	1	1	1	1	4
Bl	—	—	—	—	t	—	t	t	4
Mumps	—	—	—	—	—	—	—	—	2
Cell control	—	—	—	—	—	—	—	—	....
SECOND TRIAL (PRETREATMENT—4 HOURS AT 37 C.)									
Vic	—	—	—	—	—	—	—	t	3
Hert–E	—	—	—	—	1	t	1	2	4
Milano	—	—	—	—	2	2	1	2	4
GB	—	—	—	—	1	—	—	2	4
Roakin	—	—	—	—	—	—	—	—	2
Naj	—	—	—	—	t	—	—	1	4
Bl	—	—	—	—	t	—	—	1	4
Mumps	—	—	—	—	—	—	—	—	2
Cell control	—	—	—	—	—	—	—	—	....

* Virus and untreated red blood cells; ** —, t, 1, 2, 3, 4 = 0, trace, 25, 50, 75, and 100 per cent hemolysis, respectively.

these preparations become highly hemolytic. An immediate supposition as to the mechanism of the freeze-thaw process is that clumps or aggregates of the virus are broken up with an increase in the number of active groups. Besides disaggregation, other effects of freezing and thawing can be assumed; it is possible that the "patches" responsible for hemolysis are subsurface and that freezing and thawing bring them to an active position. It is also possible that the hemolytic patches are irreversibly united with some inhibitor and that freezing and thawing tend to dissociate the two.

Calcium ions undoubtedly have some effect on the hemolytic activity of mumps and ND viruses. The essential need of ionic constituents in any medium in order for a vast number of reactions to occur is well known. Lovell and Buckingham[6] and Davenport and Horsfall[4] showed that ions are essential for viral hemagglutination to occur. Puck *et al.*,[11] working with T phages of *Escherichia coli*, very definitely demonstrated the need of ionic constituents in the medium for the adsorption of virus to the host cell. The rate of attachment was not only dependent on concentration of ions but also on their valency, the attachment velocity being much greater with divalent ions than with monovalent ions. Differences in the rate of attachment could account for the fact that calcium favors the destruction of r.b.c. receptors[1] as well as union of virus with mucoid inhibitor.[5] Thus, if the concept of 4 types of competitive interactions between virus and red cells are correct as previously discussed, the role of calcium could well be that of hastening adsorption or increasing the efficiency of type 1 interaction, thus shortening the time required for the destruction of the receptors. This would be to the detriment of action of type 3 variant whose rate of adsorption is visualized as being much slower. In the absence of calcium ions and in the presence of monovalent ions such as Na^+, type 3 interaction would have a better chance to occur. Morgan[10] demonstrated that the divalent ions, Mn^{++} and Mg^{++}, were less effective in inhibiting hemolysis than calcium ions. This suggests that the calcium ion possesses a certain degree of specificity in the viral hemolytic reaction.

Temperature is definitely a factor in the hemolytic reaction as in other chemical and biological processes. Here again it can be assumed that in the several different types of competition for interaction, temperature may alter the kinetics of the interaction to such an extent as to favor one type to the exclusion of another type.

The hemolytic principle of mumps and ND viruses is highly sensitive to the action of heat. If hemolysis is due to a different kind of interaction than that which causes agglutination of r.b.c., then it is much more susceptible to heat. This same observation also seems to hold true for formalin sensitivity.

The hemolytic principle did not appear, in our experiments, to be as labile to storage as in the work reported by Burnet and Lind[3] and Morgan *et al.*[9] Differences in pH could account for greater stability of hemolytic activity; the pH was near neutrality in the earlier investigations; approximately 6.6 in our studies.

Results presented (table 11) suggest that a receptor gradient exists among

TABLE 12—Effect of Normal and Immune Serums on the Hemolytic Activity of Mumps and Newcastle Disease Virus Strains of Viruses

Serum	Virus	Dilution* of serum										Hemolytic control**
		5	10	20	40	80	160	320	640	1280	2560	
Mumps	Mumps	—†	—	—	—	—	—	t	1	2	2	2
Mumps	Bl	4	4	4	4	4	4	4	4	4	4	4
Mumps	Naj	4	4	4	4	4	4	4	4	4	4	4
Roakin	Mumps	1	1	1	1	1	2	2	2	2	2	2
Roakin	Roakin	—	—	—	—	—	1	2	2	2	3	3
Calif. 11914	Mumps	1	1	2	2	2	2	2	2	2	2	2
Calif. 11914	Milano	—	—	—	—	—	1	3	4	4	4	4
Normal	Naj	4	4	4	4	4	4	4	4	4	4	4
Normal	Bl	4	4	4	4	4	4	4	4	4	4	4
Normal	Milano	4	4	4	4	4	4	4	4	4	4	4
Normal No. 1	Mumps	1	1	1	1	1	2	2	2	2	2	2
Normal No. 2	Mumps	2	2	2	2	2	2	2	2	2	2	2

* In saline containing 2.0 per cent sodium citrate; ** Virus and untreated red blood cells; † —, t, 1, 2, 3, 4 = 0, trace, 25, 50, 75, and 100 per cent hemolysis, respectively.

strains of NDV. The time element appears to be extremely important and it is probable that if the reaction were allowed to proceed long enough, any strain of NDV would remove r.b.c. receptors for any other strain of NDV.

From the studies on the serological relationship of mumps and NDV hemolysin, it would appear that the mechanism of inhibition of hemolysis is not one of neutralization of the hemolytic factor by specific antihemolytic antibody but to antibody or antibodies that combine with various other receptors or parts of the virus, in the process neutralizing the entire particle to other types of interactions.

All strains of NDV were hemolytic but the degree of hemolysis produced was highly variable and apparently dependent upon factors other than particle concentration. Furthermore, the degree of hemolysis produced by these viruses could not be correlated with other of their known properties such as pathogenicity and heat stability of the hemagglutinative activity.

Summary

Studies were made of the hemolytic activity for chicken red blood cells (r.b.c.) of allantoic fluid preparations of mumps and Newcastle disease virus.

1) Untreated allantoic fluid virus, frozen and thawed allantoic fluid virus, as well as dialyzed, allantoic fluid virus produced weak hemolysis of r.b.c. Dialyzed, allantoic fluid viruses which had been frozen and thawed were highly hemolytic. Normal allantoic fluid and allantoic fluid infected with Lee strain of influenza B virus and treated in the same manner did not produce hemolysis. All strains of Newcastle disease virus (NDV) tested had the capacity to hemolyze red blood cells.

2) Saline or sodium citrate in saline provided excellent mediums for the hemolytic reaction; whereas, with calcium in saline as the diluent, an inhibitory action on hemolysis was observed.

3) Best hemolysis was obtained at 37 C., a greatly reduced degree at 25 C., and none at all at 4 C.

4) The hemolytic principle appeared to be relatively stable to refrigerator and freezing temperatures although it was rapidly destroyed at 56 C. Formalin caused progressive inactivation of hemolytic activity without appreciably altering the hemagglutinin titer.

5) The possibility that a receptor gradient exists among strains of NDV and mumps virus was considered.

6) Specific antiserum neutralized hemolytic activity without any evidence of cross neutralization of mumps and NDV hemolysis.

The significance of the results of these studies in relation to the hemolytic process was discussed briefly.

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The Pathology of Leucocytozoon Infection in Turkeys with a Note on Its Tissue Stages

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Literature Cited

Published reports concerning *Leucocytozoon smithi* infection in turkeys are not numerous, and a majority deal primarily with the clinical aspects of the disease. Published descriptions of pathological findings are brief and incomplete. Gross pathological observations have been reported by Stoddard *et al.*,[1] Savage and Isa,[2] Skidmore,[3] Johnson *et al.*,[4] and both gross and microscopic observations were reported by West and Starr.[5]

The terms proposed by Huff[6] in his study of the life cycle of *Leucocytozoon simondi* in ducks are used wherever applicable in the present paper. He described two types of schizonts: the "hepatic schizont," occurring in and apparently limited in size by the hepatic cell, and the "megaloschizont," a much larger one found in the heart, spleen, liver, and intestine. The term "cytomere" was used to indicate the individual formations of dense masses in the developing schizonts, and the term "merozoite" was used in designating the individual organism found in the final stage of schizogony.

A schizogonous phase in the life cycle of *L. smithi* has not been described.

Material and Methods

Seven 10-month-old turkeys infected with *L. smithi* were obtained from a farm flock in Georgia. Daily losses of 1 to 5 birds in the flock, numbering over 1,200, had been occurring since early February, 1953, about the time the breeding season began. Subsequent hematological examination of birds selected at random from the flock showed that the infection persisted until the entire flock was slaughtered in August, 1953. On the basis of the data, the infection was regarded as chronic in nature.

No pathogenic bacteria were recovered on routine postmortem bacteriological examination of the infected turkeys.

Histological examination was made of the lungs, liver, kidneys, spleen, brain, heart, intestines, pancreas, and bone marrow. Tissues from several 3-month-old turkeys were used for comparison with the infected turkeys. Representative tissue blocks from each organ were fixed in Zenker-formol (9.5/0.5) and were washed, dehydrated, and embedded in paraffin. Histological sections were cut 7 μ thick and stained with alum-hematoxylin and eosin for routine study. Duplicated sections stained with Giemsa were prepared from each organ. Several sections were made from selected organs and the following special stains employed: Mallory's phosphotungstic acid hematoxylin, Heidenhain's modification of Mallory's stain for connective tissue, Fuelgen's stain for thymonucleic acid, Gram's stain, a modification of Perls' Prussian blue reaction for hemosiderin, and Lillie's periodic acid-leuko-fuchsin.[7]

Results

The rate of infection in the turkeys, as determined by demonstrating gametocytes* in the blood spaces, histologically, paralleled that determined antemortem by hematological examination. Stages in the development of gametocytes were observed, but because of the problem of cell identification following tissue fixation, no attempt was made to determine a complete developmental series. The recognizable, mature gametocytes were elongated in all instances.

Tissue Stages.—Tissue stages were observed in the liver of only 1 of the 7 turkeys. The schizonts were within hepatic cells. Morphological changes of the invaded hepatic cell either did not occur or were very slight, inasmuch as there was no apparent distortion of either the host cell or its nucleus. Measurements of 40 hepatic schizonts showed variations in size ranging from 7.0 by 10.5 μ to 14.0 by 19.5 μ, with an average of 10.5 by 13.5 μ.

The earliest stage of hepatic schizont identified (fig. 1) showed development of round and crescent-shaped, basophilic cytomeres. A later stage (fig. 2) showed only vestiges of cytomeres which had developed into masses of merozoites which were also basophilic. The final stage (fig. 3) consisted of a homogeneous mass of deeply stained merozoites that completely filled the host cell cytoplasm. In none of the

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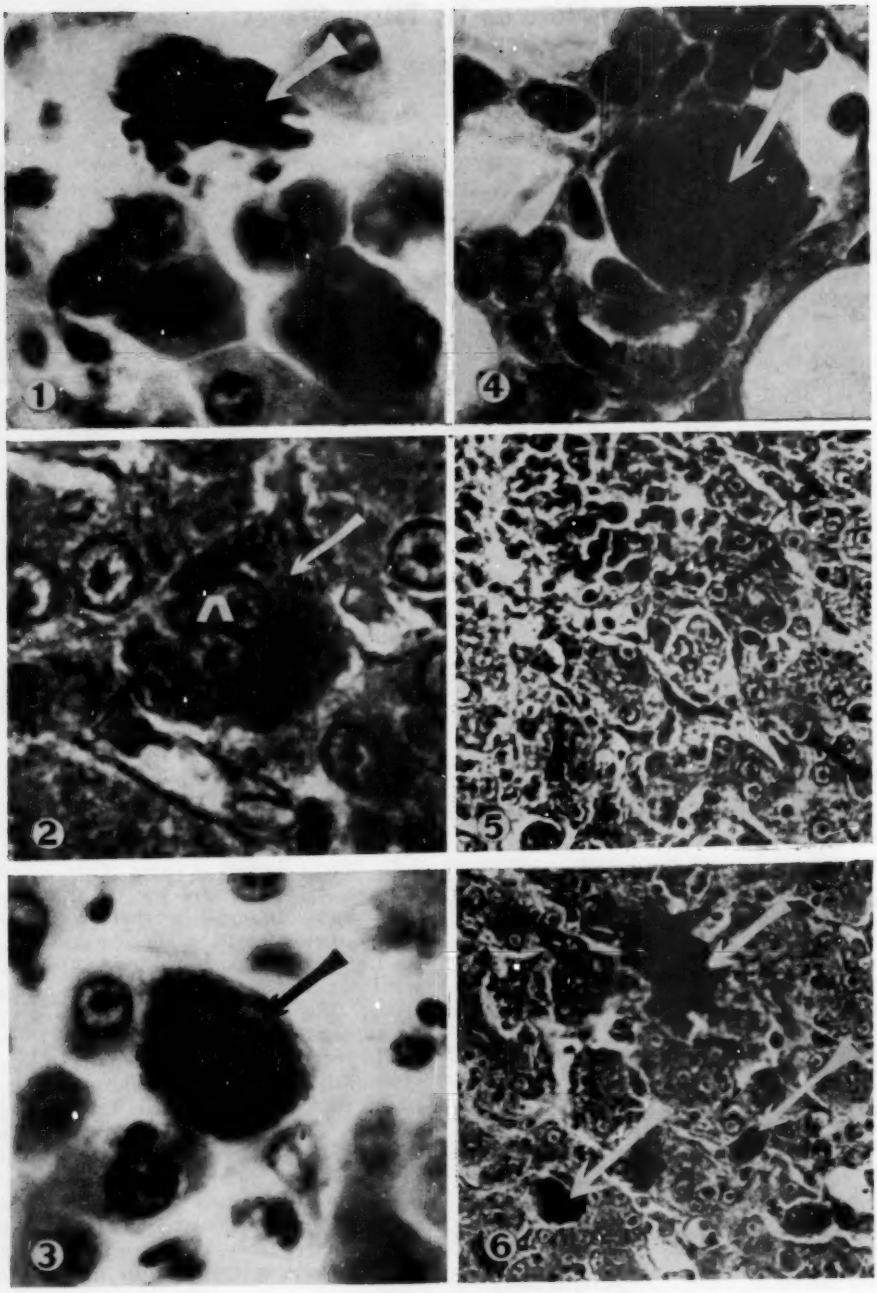
* A term commonly used to designate a stage of the parasite found in peripheral blood.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three stages was there any appreciable alteration of the host cell nucleus. Several schizonts were observed that had ruptured or disintegrated, allowing the merozoites to escape into the surrounding sinusoids.

Megaloschizonts were not found in any of the 7 infected turkeys.

Several unidentified, small, basophilic bodies were seen within macrophages in the hepatic sinusoids. These bodies resembled merozoites, morphologically, but generally there were fewer within each cell than was the case with a hepatic schizont. In some instances, the macrophage containing these bodies was located near a ruptured hepatic schizont. One unidentified spherical body (fig. 4) measuring 23 μ in diameter and containing crescent-shaped bodies, morphologically similar to cytomeres, was observed in the bone marrow of 1 turkey.

Lesions Produced by *Leucocytozoon Smithi*.—A) Local Host Tissue Reaction to the Parasitized Cells.—Gametocytes were seen in greatest concentration in the lungs, liver, and spleen. Although capillaries of these organs were, in many instances, markedly distended by the presence of these parasites, there was no histological evidence of local host tissue reaction to gametocytes in any of the turkeys. The parasitized hepatic parenchymal cells (hepatic schizonts) failed to elicit any apparent host tissue reaction.

B) Pathological Alterations Associated with Systemic Infection.—(1) Liver.—Similar pathological alterations were observed in the livers of all the infected turkeys. There was moderate to marked increase of lymphocytes, both focal and diffuse in distribution (fig. 5). The focal lymphocytic infiltration consisted of numerous small aggregates of lymphoid cells scattered through the liver in both periportal and intralobular areas. The diffuse lymphocytic infiltration was evident in both the sinusoids and the hepatic cell cords. Numerous individual lymphocytes had penetrated the sinusoidal walls and had become wedged between hepatic parenchymal cells. Many of the invading lymphocytes were pyknotic, and their cytoplasm was shrunken to form an eccentric pink-staining globular mass. Other lymphocytes that had penetrated the sinusoidal walls had not undergone pyknosis, and their identity was readily established.

Many of the Kupffer's cells were swollen and contained a mixture of phagocytized cellular debris and amorphous particles of golden brown pigment. A considerable amount of the pigment gave a positive histochemical reaction for hemosiderin (fig. 6).

2) Other Organs.—The spleen of only 1 turkey showed pathological alteration, which consisted of minimal macrophage proliferation and deposition of pigment in these cells. The lungs of several turkeys showed various degrees of congestion, and in 1 bird the bone marrow was moderately hyperplastic. Pathological alterations were not seen in the brain, heart, intestine, pancreas, or kidneys.

Discussion

The gametocytes in the smears of peripheral blood and in the blood spaces of histological sections were elongate forms. There are no reports presently available which indicate that mature round gametocytes have ever been seen either in acute or

Legends for Illustrations on Opposite Page

Fig. 1—Section of turkey liver showing a partially developed hepatic schizont (arrow). Note the individual, large, dense masses (cytomeres) developing in the cytoplasm. Stain, hematoxylin and eosin. x 2,000.

Fig. 2—Section of turkey liver showing late development of a hepatic schizont (arrow). Note the unaltered double nucleus of the parasitized parenchymal cell (A). Stain, hematoxylin and eosin. x 2,350.

Fig. 3—Section of turkey liver showing a completely developed hepatic schizont (arrow). Note the small, dense masses (merozoites) in the cytoplasm and the unaltered host cell nucleus. Stain, hematoxylin and eosin. x 2,000.

Fig. 4—An unidentified spherical body (arrow) found in the bone marrow of a turkey. Stain, hematoxylin and eosin. x 1,300.

Fig. 5—Section of turkey liver showing lymphocytic infiltration. Note individual lymphocytes (arrow) within hepatic cords. Stain, hematoxylin and eosin. x 480.

Fig. 6—Section of turkey liver showing enlarged Kupffer's cells (arrows) which contain hemosiderin. Stain, Prussian blue. x 390.

chronic *L. smithi* infection. This is in contrast to the descriptions by other workers [6,8-11] of both round and elongate forms in *L. simondi* infection. These two forms were considered by some workers [6,11] to represent different stages in the maturation process. However, Fallis *et al.*[9] have reported exflagellation of both round and elongate microgametocytes, and their observation was subsequently confirmed by Rawley [10] who also observed exflagellation and maturation of both forms and concluded that both forms were mature.

West and Starr [5] reported seeing one schizont in the intestinal mucosa of a turkey infected with *L. smithi*, but this is the first illustrated description of a schizogonous phase of this parasite. Hepatic schizonts were demonstrated in 1 of the 7 infected turkeys only after a most thorough search of the material. The schizonts developed within hepatic parenchymal cells. The difficulty encountered in demonstrating hepatic schizonts may have been due partially to their small size, which averaged 10.5 by 13.5 μ. In several instances, for example, hepatic schizonts were demonstrated in certain sections but not in either of the adjacent serial sections. It follows that hepatic schizonts may have been present in additional birds as well but not in sufficient numbers to be detected.

Megaloschizonts were not demonstrated in any of the turkeys with *L. smithi* infection. The observation that two forms of mature gametocytes and two types of schizonts occur in *L. simondi* infection, whereas only one form of mature gametocyte and one type of schizont has been found in *L. smithi* infection, raises an interesting question regarding the relationship of these various forms, especially those of *L. simondi*. Huff [6] suggested three hypotheses regarding the relationship between the hepatic schizont and megaloschizont of *L. simondi:* (1) the two types of schizonts may be different stages in the same life cycle; (2) they may be essentially similar stages except for location, with those entering liver cells being limited in size by the type of host cell; or (3) they may represent stages in the life cycle of two different species of parasites. The present study does not aid significantly in resolving this question, and there is not sufficient evidence now available to establish conclusively any of the hypotheses as correct.

The absence of local host tissue reaction to the gametocytes and hepatic schizonts is not surprising, since both stages of the parasite are intracellular. The systemic histopathological alterations attributable to the infection showed close correlation with the clinical severity and duration of the disease and, in general, reflected its chronic nature. The hemosiderosis of the spleen and liver can be attributed to chronic red blood cell destruction. The lymphocytic infiltration seen in the liver was apparently due to general stimulation of the lymphoid system by the infection.

Cook [8] has presented evidence to show that the host cell of the mature gametocyte stage in *L. simondi* of ducks is the erythrocyte only; Fallis [9] has recorded an extreme anemia in ducks associated with severe *L. simondi* infection. Although these results can not be compared to *L. smithi* infection in turkeys, it may be postulated that the chronic red blood cell destruction in the turkeys, as evidenced by hepatic hemosiderosis, may have resulted from the presence of the parasite in erythrocytes. The fact that none of the birds show a clinically apparent anemia further supports the view that the disease in the turkeys was chronic.

The lymphocytic infiltration seen in the turkeys was not considered a specific manifestation of *L. smithi* infection but rather a general reaction of the lymphoid system to the disease process.

SUMMARY

1) Histopathological studies were made of various organs of 7 turkeys with chronic infection with *Leucocytozoon smithi.*

2) Mature gametocytes of *L. smithi* found in the peripheral blood and tissue blood spaces were elongate forms.

3) Schizonts of *L. smithi* were found only in the liver and were of the type designated "hepatic schizonts" in *Leucocytozoon simondi* infection. This is the first illustrated description of a schizogonous phase of *L. smithi.*

4) Local host tissue reaction did not occur in relation to the gametocyte or hepatic schizont stages of the parasite.

5) Significant pathological alterations resulting from chronic systemic infection

were hepatic hemosiderosis and lymphocytic infiltration.

6) Minor, but variable, pathological alterations occurred in the spleen, lungs, and bone marrow.

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Studies on Pasteurellosis. I. A New Species of Pasteurella Encountered in Chronic Fowl Cholera

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IN THE course of examining two flocks of chickens which were suffering from chronic fowl cholera, an atypical Pasteurella organism was isolated from the upper respiratory tracts of approximately 50 per cent of the birds cultured. This organism has been repeatedly recovered from the nasopharynx and conjunctiva of birds in these flocks during the past four years. Biochemically, serologically, and in other ways, it differs from *Pasteurella multocida* and *Pasteurella hemolytica.*

No extensive review of the literature is contemplated at this time as it is comprehensively reviewed by Rosenbusch and Merchant.[11] Jones[5] was one of the first to classify Pasteurella using 16 strains from cows and calves suffering with pneumonia. Although all 16 strains were from one species of animal, it was necessary to divide them into three groups in order to classify them biochemically and serologically.

Jones' Classification of Pasteurella

Group	Dextrose	Lactose	Sucrose	Maltose	Mannitol	Indol	Hemolysis	Bile solubility	
1	+	+	+	+	+	−	+	−	atypical (*P. hemolytica*)
2	+	−	+	−	−	+	−	+	typical or classic (*P. multocida*)
3	+	−	+	−	+	+	−	−	

Newsom and Cross[10] classified 20 strains of Pasteurella from cases of pneumonia in sheep and cattle. These were divided into two main groups, *viz.*: (1) typical or classical *(P. multocida)* which corresponded to Jones' group 3 and (2) atypical, which corresponded to Jones' group 1. They made their classification into typical and atypical largely on the basis of indol production, hemolysis, and pathogenicity for rabbits as these characters seemed so clearly marked and unchangeable. Because of the marked differences, they suggested a new species name, "*Pasteurella hemolytica,*" for Jones' atypical group 1. Hughes[8] suggested a classification of Pasteurella of poultry based on the optical characteristics of the colonies, *viz.*, (1) fluorescent—this type is associated with epizootics of fowl cholera and is highly virulent, stable in suspension, and inagglutinable in anti-fowl cholera serums; (2) blue—this type is associated with enzootics of fowl cholera, is low in virulence, unstable in suspension, and agglutinates readily in anti-fowl cholera serums; (3) intermediate—this type is intermediate in its behavior. The fluorescent may develop intermediate characteristics after prolonged cultivation on artificial mediums. These optical characteristics may be readily observed by placing a Petri plate of well-scattered colonies on the glass stage of a wide-field dissecting-type scope and using oblique transmitted illumination from the under side of the stage.

Schneider[13] classified 373 strains of Pasteurella into 8 groups. Atypical group 7 included three poultry strains and three swine strains. The basis for classification of all strains was their acid-producing ability in arabinose, xylose, and sorbitol. The three poultry and three swine strains found in group 7 corresponded rather closely with our atypical Pasteurella organism except for trehalose which was negative with Schneider's strains and positive with our organism.

Pasteurella have been isolated from a number of animal species, some individuals of which were apparently normal while others showed respiratory inflammation. Moore[9] reported finding virulent Pasteurella in 80 per cent of cattle and 48 per cent of apparently normal hogs. Jorgenson[6] made a study of the upper respiratory tract of 250 normal cattle and isolated *Pasteurella bovisceptica* from 37 (15 %). They were found to be of four types, three of which coincided with types isolated by Jones.[5] Johnson[4] swabbed the throats of 11 normal birds but failed to recover any Pasteurella. However, culture of 12 birds with roup yielded *Pasteurella avicida* from 7. Gibbs[1] described an organism isolated from ducks which, on injection, caused death of rabbits but not of guinea pigs. The organism was said to resemble *P. avicida.* However, it was maltose positive and indol negative. Schipper[12] cultured 102 wild rats and isolated virulent *P. multocida* from the throats of 14. Eleven strains of Pasteurella, identified as *P. mastitidis* and isolated from the udders of sheep, were recently received from Marsh.[8] After biochemical, cultural, and serological tests, we found them to be indistinguishable from *P. hemolytica.* In a study of the relationship be-

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tween *P. multocida* isolations and atrophic rhinitis in 172 swine varying in age from weanlings to adults, one of us (K.L.H.)[2] made ten isolations of *P. multocida*, six from rhinitis-positive swine, and four from rhinitis-negative swine.

Materials and Methods

During 1950, 576 laying fowl were acquired from two institutional flocks. From flock 1, 400 White Leghorns showing signs of cholera were obtained. In this flock, which consisted of 10,000 layers, fowl cholera had been enzootic for many years. About 3,000 of these layers were yearlings which were carried over from the previous year for breeding purposes. Each year in November or December, the pullet flock developed coryza and also some swollen wattles. During the winter, morbidity was very high and mortality averaged approximately 10 per cent. The outbreak continued with scattered mortality until spring with *P. multocida* regularly isolated on necropsy. From flock 2, 176 birds, mostly White Leghorns and a few Rhode Island Reds affected with chronic fowl cholera, were studied.

During the next four years, birds from these two flocks were cultured 16 different times for the purpose of determining the prevalence and significance of the various species of Pasteurella.

The conjunctiva and the palatine cleft were swabbed with cotton on a wood applicator. The swabs were placed into tubes of serum broth and after overnight incubation the cultures were plated on a modified MacConkey's agar from which the bile salts had been omitted and to which had been added about 7.5 per cent horse serum. Dextrose starch agar, without serum, and beef infusion agar with 7.5 per cent chicken blood serum have now supplanted modified MacConkey's agar.

Additional field studies were made on a large breeding farm to determine the incidence of *P. multocida* and the atypical Pasteurella organism in extensive respiratory disease outbreaks then prevalent in their laying flocks. It is possible that several disease entities such as Newcastle disease, infectious bronchitis, infectious coryza, chronic respiratory disease, and Pasteurella infection may have entered into the respiratory complex in these flocks.

Approximately 5,000 layers in house 1 came down with a respiratory disease characterized by nasal discharge, swollen head, râles, cough, loss of flesh, diarrhea, and a sharp decline in egg production. The condition also affected six other houses of similar capacity. The malady was characterized by high morbidity and a mortality of approximately 10 per cent. The flock had been vaccinated against Newcastle disease at 1 day of age and at 5 weeks of age with the intranasal B_1 vaccine and the wing web vaccine, respectively. Vaccination against laryngotracheitis was carried out also at the time of the second Newcastle vaccination. The nasopharynx and conjunctiva of 57 birds were cultured at random in this house.

In laying house 2, four pens of birds showing various stages of respiratory disease, from inapparent through acute and chronic, were cultured by swabbing the nasopharynx and conjunctiva.

In reporting an apparently new species of Pasteurella in poultry, consideration should also be given to other domestic animals since the various types of Pasteurella are said to cross host species lines.[7, 11, 16] Hence, it was considered desirable to study the species of Pasteurella found in livestock. A total of 382 large animals were cultured as shown in table 5. All of the cattle cultured were healthy young steers with the exception of 5 old cows. The steers were cultured by swabbing the nasal cavities after slaughter. Some cultures were also made from the bronchi and mediastinal lymph glands. The sheep and swine were also cultured by swabbing the nasal cavities, mostly at necropsy.

The fermentable broth medium used in this

TABLE 1—Pasteurella Cultures Isolated from the Conjunctiva and Nasopharynx of Live Birds Affected with Chronic Fowl Cholera, Flocks 1 and 2

Date cultured	Pen (No.)	No. of birds cultured	Cultures isolated			
			Conjunctiva		Nasopharynx	
			P. n.sp.	P.M.	P. n.sp.	P.M.
8–3–50	24	37	2	0	6	0
2–13–51	24	85	13	2	20	2
3–5–51	19A	21	1	2	2	1
4–24–51	19B	38	10	2	16	1
5–15–51	20A	57	14	4	24	0
6–4–51	20B	49	12	0	16	2
7–30–51	23	72	23	0	28	0
11–14–51	23	60	25	1	32	3
12–4–51	24	67	12	2	37	2
3–24–52	20	56	16	0	24	0
4–9–52	19	30	10	1	17	0
4–23–52	19	37	20	0	25	1
2–4–53	20	104	54	13	4	2
6–30–53	20	104	14	0	39	0
11–18–53	20	97	2	0	7	5
6–8–54	20	78	5	4	22	10
Total		992	233	31	319	29

P.M.—*P. multocida*; P. n.sp.—Pasteurella n. sp.

study (table 3) was prepared according to the following formula.

Beef extract	5 Gm.
Peptone (Difco)	10 Gm.
Sodium chloride	5 Gm.
Carbohydrate or alcohol	10 Gm.
Andrade indicator	
Distilled water q.s.	1,000 cc.

Serum was not added because *P. multocida* will ferment dextrin and maltose with serum but will not when serum is omitted. The Gore test was used for the detection of indol.

Serological work over the past several years on *P. multocida* and the atypical Pasteurella organism has consisted of agglutination tests, both tube and rapid plate. In the plate tests, both the serum and whole-blood methods were used. The final dilution of serums used in the plate test was 1:10.

Tube agglutinating antigens were produced by washing off the growth from 48-hour slant cultures with physiological saline containing 0.5 per cent phenol. Antigens were adjusted to No. 2 (McFarland) density. Intermediate (Hughes' classification) colonies were selected for antigen production. Plate antigens were made in a similar manner except they were adjusted to No. 25 (McFarland) density.

Antiserums were produced with live cultures of the atypical Pasteurella organism. Eight intravenous injections were made in year-old New Hampshire pullets at intervals of three to five days. The dose was increased from 0.5 ml. of No. 4 to 1.0 ml. of No. 25 (McFarland) density. The same method was used for *P. hemolytica* except there were ten injections. Anti-*P. multocida* serums were produced with 26 injections over a period of fourteen weeks. Killed bacteria were used for the first 18 intravenous injections, and the dosage was increased from 0.25 to 1.75 ml. of No. 4 (McFarland) density. Living bacteria were used for the last eight intravenous injections, and the dosage was increased from 0.1 to 1.0 ml. of No. 1 (McFarland) density. All birds were bled seven days after the last injection. Serums were preserved with 0.02 per cent merthiolate.

For the tube agglutination test, twofold serial dilutions of the antiserum were made in 12 by 75 mm. tubes. To each tube containing 0.5 ml. of diluted serum was added 0.5 ml. of antigen suspension. Two control tubes were set up for each antigen as follows: (1) 0.5 ml. saline and 0.5 ml. of antigen suspension; (2) 0.5 ml. of 1:10 normal chicken serum and 0.5 ml. of antigen suspension. The tubes were shaken and held overnight in a 37.5 C. hot air incubator. After incubation the results were read and recorded according to the degree of agglutination.

RESULTS

The atypical Pasteurella organism mentioned at the beginning of the paper will be identified in the tables and text to follow as "Pasteurella n. sp.," or "P. n. sp."

Much of the data on which this paper is based will be found in tables 1, 2, 3, and 4. Table 1 shows the results of cultures from the nasopharynx and conjunctiva of live birds in the two flocks affected with chronic fowl cholera. The number of isolations of Pasteurella n. sp. is far greater (90.1 %) than the number of isolations of *P. multocida* (9.9 %). More cultures of *P. multocida* and Pasteurella n. sp. were obtained from the nasopharynx (56.9 %) than from the conjunctiva (43.1 %).

Table 2 shows that the relative number of isolations of *P. multocida* (79.9 %) and Pasteurella n. sp. (20.1 %) at necropsy is reversed as compared with those from the conjunctiva and nasopharynx of live birds in table 1.

In table 2 attention is also called to the relative frequency of recovery of the respective organisms, *P. multocida* and Pasteurella n. sp. from different organs, as shown by the following tabulation:

Order of frequency	*Pasteurella multocida*	*Pasteurella n.sp.*
1	Liver	Conjunctiva
2	Lung, marrow	Nasopharynx
3	Pericardial fluid	Liver
4	Gonads	Lung
5	Kidney, nasopharynx	Gonads
6	Conjunctiva	Marrow
7	Spleen	Pericardial fluid
8	Wattle	Kidney, spleen

TABLE 2—Comparative Frequency of Isolation of Two Species of Pasteurella from Various Organs in Chronic Cases of Fowl Cholera

	Conjunctiva	Nasopharynx	Liver	Pericardial fluid	Spleen	Lung	Gonads	Marrow	Kidney	Wattle	Other	Total	Per cent
P. multocida													
No.	16	22	49	26	13	36	23	36	22	8	7	258	79.9
%	6.2	8.5	18.9	10.07	5.03	13.95	8.91	13.95	8.52	3.10	2.71		
Pasteurella n.sp.													
No.	11	10	9	5	3	8	7	6	3		3	65	20.1
%	16.92	15.38	13.84	7.69	4.61	12.30	10.76	9.23	4.61	.....	4.61		
Total												323	

TABLE 3—Differential Characteristics of Three Species of Pasteurella*

	pH of mediums before inoculation	*P. hemolytica*								*P. multocida*								Pasteurella n. sp.							
		2976	P-779	3684	3680	3678	3679	3681	3682	P-27	P-32	P-206	P-705	P-765	P-798	P-763	P-500	P-626	P-516	P-552	P-632	3597	3616	3617	3634
Dextrose	7.00	6.00	4.91	5.94	5.73	6.06	5.80	5.78	6.05	5.52	5.68	5.78	5.75	5.58	5.61	5.45	5.80	5.60	5.30	5.37	5.10	5.38	5.40	5.30	5.30
		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Lactose	7.00	6.14	4.70	6.30	6.06	6.15	6.12	6.20	6.15	7.20	7.00	7.13	6.75	7.02	7.08	7.02	6.90	7.30	6.89	6.89	6.89	7.02	6.90	7.05	6.95
		+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Sucrose	7.10	6.00	5.80	5.96	5.80	5.94	5.82	6.00	5.75	5.60	5.70	5.71	5.68	5.68	5.70	5.45	6.02	5.55	5.35	5.49	5.06	5.39	5.45	5.33	5.36
		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Maltose	7.05	6.00	4.98	6.00	5.84	5.90	5.92	5.75	5.76	6.95	6.75	6.90	6.95	7.18	7.10	6.75	6.75	5.52	5.58	5.45	5.15	5.45	5.45	5.45	5.50
		+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+
Arabinose	6.70	6.45	6.42	6.62	6.40	5.96	6.45	6.08	6.40	6.42	6.60	6.72	6.50	6.60	6.50	6.35	6.72	6.66	6.65	6.60	7.00	6.79	6.70	6.80	6.73
		±	±	−	±	+	±	+	±	±	−	−	−	−	−	±	−	−	−	−	−	−	−	−	−
Dulcitol	7.10	6.95	6.95	6.72	6.82	6.70	6.66	6.70	6.60	7.22	5.80	7.11	5.78	6.85	7.15	7.05	7.00	6.95	6.89	6.69	6.70	7.00	7.05	7.00	7.29
		−	−	−	−	−	−	−	±	−	+	−	+	−	−	−	−	−	−	−	−	−	−	−	−
Xylose	6.70	6.18	5.96	5.90	6.20	6.14	6.00	6.04	5.92	5.65	6.70	6.00	6.50	5.85	5.91	6.55	6.40	6.85	6.62	6.51	6.55	6.72	5.65	6.80	5.58
		+	+	+	+	+	+	+	+	+	−	+	−	+	+	−	±	−	−	−	−	−	+	−	+
Trehalose	7.15	7.22	7.43	6.98	7.10	6.94	6.98	7.20	6.75	7.20	7.11	7.10	7.25	7.02	7.32	7.18	6.90	5.52	5.52	5.54	5.10	5.51	5.40	5.50	5.32
		−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+
Dextrin	7.15	6.30	5.10	6.02	5.94	6.20	6.02	5.95	5.95	7.22	7.20	6.90	7.40	7.15	7.40	7.12	7.80	5.50	5.93	5.54	5.25	5.61	5.45	5.41	5.42
		+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+
Mannose	7.00	6.87	6.92	6.84	6.85	6.90	6.78	6.88	6.73	5.63	5.20	5.80	5.68	5.70	5.76	5.50	6.00	5.50	5.35	5.40	5.10	5.33	5.84	5.31	5.58
		−	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Hemolysis		+	+	−	−	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Indol		−	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−
MacConkey's agar		+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

* Andrade's indicator and a pH meter used to determine acid production; + = acid; − = no acid; ± = questionable.

Among the cultures from the conjunctiva, nasopharynx, liver, and gonads, both species of Pasteurella were isolated from the same organs in the same bird, one, three, two, and one times, respectively. Table 2 represents the total cultures from 163 birds. Of these, 117 birds yielded 225 cultures of *P. multocida* only; 23 birds yielded 30 cultures of Pasteurella n. sp. only, and 23 birds yielded 68 cultures of both species of Pasteurella.

Characterization of Pasteurella n. sp.— Biochemical and cultural studies were made of 52 strains of Pasteurella of which 24 are shown in table 3. These strains were selected from three species of Pasteurella, *viz.*: *P. hemolytica*, *P. multocida*, and Pasteurella n. sp.

The first column of figures on the left (table 3) gives the pH reading of each sugar medium before inoculation. On the line opposite each sugar are the pH readings for acid production as determined with the Beckman pH meter. Immediately below these figures are the corresponding readings as determined by the color change of the Andrade indicator. The pH meter readings were made to ascertain the accuracy and consistency of the color change readings. In general, these two readings

TABLE 4—Cross-Agglutination Tests of Three Species of Pasteurella

Antigens*	Antiserums	Dilutions of antiserums						
	P. multocida	1:20	1:40	1:80	1:160	1:320	1:640	1:1280
P. multocida	32	4**	4	4	4	4	3	1
	27	4	4	4	4	2	—	—
	X73	4	4	4	4	4	3	—
	705	3	4	4	4	4	3	2
	Pasteurella n. sp.							
	828	1	—	—	—	....	....	....
27	3005	—	—	—	—	....	....	....
	892	2	—	—	—	....	....	....
	833	—	—	—	—	....	....	....
	P. hemolytica							
	823	1	—	—	—	....	....	....
	829	—	—	—	—	....	....	....
	830	1	—	—	—	....	....	....
	777	—	—	—	—	....	....	....
Pasteurella n. sp.	*P. multocida*							
	32	—	—	—	—	....	....	....
	27	—	—	—	—	....	....	....
	X73	—	—	—	—	....	....	....
	705	—	—	—	—	....	....	....
892	Pasteurella n. sp.							
	828	4	4	4	4	2	—	—
	3005	4	4	4	4	3	—	—
	892	4	4	4	4	3	2	1
	833	4	4	4	4	2	1	—
	P. hemolytica							
	823	2	—	—	—	....	....	....
	829	—	—	—	—	....	....	....
	830	—	—	—	—	....	....	....
	777	—	—	—	—	....	....	....
P. hemolytica	*P. multocida*							
	32	1	—	—	—	....	....	....
	27	—	—	—	—	....	....	....
	X73	—	—	—	—	....	....	....
	705	—	—	—	—	....	....	....
	Pasteurella n. sp.							
	828	—	—	—	—	....	....	....
777	3005	—	—	—	—	....	....	....
	892	—	—	—	—	....	....	....
	833	—	—	—	—	....	....	....
	P. hemolytica							
	823	4	4	4	4	3	1	—
	829	4	4	3	1	—	—	—
	830	4	4	4	4	4	4	2
	777	4	4	4	4	4	3	2

* Controls using normal serum and physiological saline were negative.

** 4 = Agglutination in clear medium; 3 = agglutination in slightly cloudy medium; 2 = agglutination in cloudy medium; 1 = slight agglutination in cloudy medium; — = no agglutination.

were fairly consistent although the color change was difficult to interpret in a few instances.

The distinguishing characteristics of the three Pasteurella species are presented in considerable detail (table 3). However, the following tabulation presents briefly the important differential characteristics.

Differential Characteristics of Three Species of Pasteurella

	P. hemolytica	*P. multocida*	Pasteurella n.sp.
Lactose	+	−	−
Maltose	+	−	+
Trehalose	−	−	+
Mannose	−	+	+
Hemolysis	+	−	−
Indol	−	+	−
MacConkey's agar	+	−	−

Other biochemical reactions of the new species not included in table 3 were as follows: acid but not gas was produced in galactose, raffinose, and levulose. Acid was not produced in rhamnose, inulin, glycerol, and salicin. Acid production was irregular in mannitol, sorbitol, and inositol.

On serum agar or dextrose starch agar the colonies are fluorescent, circular, smooth, entire, convex, and translucent. There was good growth on beef infusion agar and a heavy growth on dextrose starch agar or on infusion agar with serum. There was no growth on unmodified MacConkey's agar. It is an aerobe and faculative anaerobe. After prolonged cultivation on artificial mediums, the fluorescent form may be succeeded by intermediate or blue forms. However, fluorescence was more persistent in Pasteurella n. sp. than in *P. multocida*.

It is an encapsulated gram-negative rod, and when isolated from acute cases it exhibits bipolar staining. Bipolarity may disappear after culture on laboratory mediums but may reappear after chicken embryo or animal passage. The cells usually occur singly but sometimes dissociate into filamentous forms when maintained on laboratory mediums.

Pathogenicity was low. Out of many chickens and rabbits inoculated, only occasionally did either one die. Chicken embryos died within twenty-four hours after inoculation.

Serology.—Serological work over the past several years on *P. multocida* and Pasteurella n. sp. has consisted of agglutination tests, both tube and rapid plate. In the plate tests both the serum and whole-blood methods were used. Some typical cross-agglutination tube tests of three species of Pasteurella, *viz.*, *P. multocida*, Pasteurella n. sp., and *P. hemolytica*, are shown (table 4). From the serological work done thus far, it appears that these three species of Pasteurella are serologically distinct.

Field studies indicated the presence of the new species of Pasteurella in considerable numbers in connection with outbreaks of respiratory disease. Of 57 birds cultured at random in laying house 1, 34 or 59 per cent harbored Pasteurella n. sp. In laying house 2, where the disease was noted in four stages from inapparent to chronic, the average recovery of Pasteurella n. sp. was 15.9 per cent.

No Pasteurella species have been recovered from the upper respiratory tract of approximately 400 birds from our breeding flock which to our knowledge have not been exposed to the organism. These birds were cultured at various times over a period of several years.

Table 5 shows the results of culturing the nasal passages and other organs of large animals at necropsy. Of 35 isolations of three species of Pasteurella, three were Pasteurella n. sp., two being isolated from cattle and one from a sheep.

Discussion

The true significance of this new species of Pasteurella in the development of res-

TABLE 5—Results of Culturing the Nasal Passages and Other Organs of Large Animals at Necropsy

Species	No. cultured	Pasteurella recovered (No.)	(sp.)	(%)
Cows	5	1	*P. multocida*	60.0
		2	Pasteurella n.sp.	
Steers	76	0		
Swine				6.4
Adult	11	2	*P. multocida*	
Pigs	144	8	*P. multocida*	
Sheep	144	4	*P. multocida*	15.9
		18	*P. hemolytica*	
		1	Pasteurella n.sp.	
Goats	6	0		

All of the above animals were healthy except that some of the hogs had atrophic rhinitis.

piratory and septicemic diseases of poultry is yet to be determined. This organism has been isolated consistently and more often than *P. multocida* from live birds in flocks suffering with chronic fowl cholera, as well as from flocks suffering with undiagnosed respiratory disorders usually initiated with a coryza. No claim is now made, however, that this newly described species of Pasteurella has etiological significance in the development of either fowl cholera or the upper respiratory diseases. Moreover, this new species of Pasteurella has not been isolated from outbreaks of acute fowl cholera where the disease is not accompanied by coryza. Further work needs to be done to determine its etiological role in diseases initiated by coryza-like conditions. Owing to the frequency with which the new species of Pasteurella was encountered in studying chronic fowl cholera, it was considered desirable to describe and identify the organism so as to avoid confusion in the present and future studies of the Pasteurella.

SUMMARY AND CONCLUSIONS

A bipolar gram-negative nonmotile microorganism which has the attributes of a Pasteurella is reported. It ferments most carbohydrates with moderate production of acid but not gas. It is fluorescent colonially when isolated but may become intermediate or blue after prolonged cultivation on artificial mediums. It does not produce indol, is nonhemolytic, and is low in virulence. This new species differs biochemically, serologically, and in growth characteristics from the other two species of Pasteurella commonly found in livestock, *viz.*, *P. multocida* and *P. hemolytica*. It was isolated from chickens suffering from chronic fowl cholera, and also from chickens with some other maladies characterized by inflammation of the upper respiratory tract. The organism was not isolated from healthy fowls. On the other hand, the authors, as well as a number of other investigators of the Pasteurella problem, have isolated both *P. multocida* and *P. hemolytica* from the upper respiratory tract of apparently normal cattle and sheep, as well as from those exhibiting catarrhal inflammation of that tract. Of 386 large animals cultured from the upper respiratory tract, 3 yielded cultures of the new species of Pasteurella; two from cattle and one from a sheep.

In conclusion, this newly described microorganism resembles the Pasteurella but differs from the other members of this genus commonly found in poultry and livestock, *viz.*, *P. multocida* and *P. hemolytica*, and is most frequently isolated from poultry. Our suggestion, therefore, is that it be designated as a new species of Pasteurella, and that it be named *Pasteurella gallinarum*.

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A Strain of Micrococcus Exhibiting Antihemolytic Properties

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THE PURPOSE of this paper is to describe the unique properties of a Micrococcus which was isolated from a milk sample examined in the course of our investigations on mastitis. The fortuitous occurrence of this strain in conjunction with an α-β Micrococcus on a blood agar plate displayed a marked inhibiting effect on the β zone of hemolysis of the latter organism. It was felt that this phenomenon merited further investigation.

The inhibitory strain did not exhibit hemolysis of its own; it was a gram-positive Micrococcus which did not produce coagulase. Fermentation reactions disclosed utilization of glucose and maltose; no change was observed in manitol, lactose, sucrose, glycerol, salicin, raffinose, inulin, or litmus milk. The organism was negative to methyl red dye, to Voges-Proskauer reaction and did not produce indole nor liquefy gelatin.

Streaking the isolated culture perpendicularly to a stock culture of β hemolytic *Micrococcus pyogenes* demonstrated the marked inhibition of the hemolysis in adjacent areas (fig. 1). The inhibition was persistent after refrigeration. This ability to inhibit hemolysis was apparently increased (as observed on plate culture) by inoculating the organism into a bacteriologically clean cow's udder and reisolating after twenty-four to forty-eight hours. The organism could be isolated from the gland for periods exceeding twenty-one days. Udder inoculation was followed in twenty-four hours by an acute inflammatory response.

The inhibitory effect against the action of Micrococcus β hemolysin, and to some degree against that of the α hemolysin, suggested the possibility of a substance that either neutralized these hemolytic toxins or that acted on the red cell membrane and thus prevented hemolysis. With this in mind, titration experiments were carried out pitting the inhibitory substance against the various hemolytic toxins of *M. pyogenes*.

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MATERIALS AND METHODS

Toxin Titration.—Stock cultures of *M. pyogenes* that produced α or β hemolytic toxin, as well as a pure culture of the inhibitory strain, were inoculated into separate flasks of modified Walbum medium prepared for toxin production.* These cultures were agitated continuously for forty-eight hours of incubation. The resultant products were centrifuged and the supernatant fluids were preserved for toxin titrations.

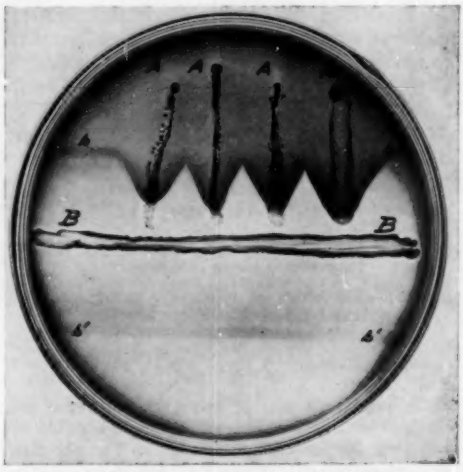
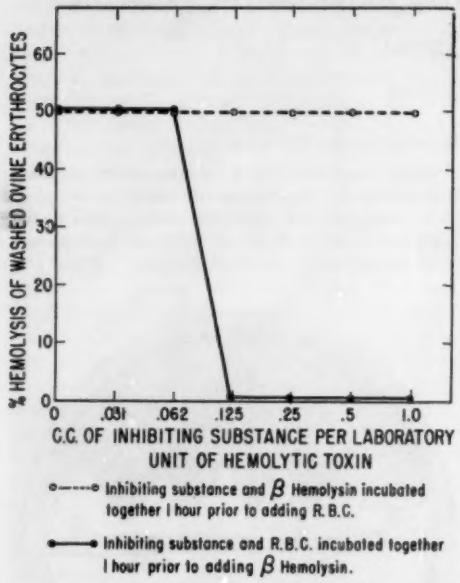


Fig. 1—Inhibitory micrococci (A) streaked perpendicularly to a stock culture of hemolytic Micrococcus pyogenes (B). The V-shaped zones dipping into the broad zone (b to b') of β hemolysis demonstrates the inhibiting effect on β hemolysis.

The hemolytic toxins thus prepared were serially diluted with saline and the titrations carried out much as described by Roy.[1] To the dilutions of hemolytic toxins, triple-washed sheep or rabbit cells were added until a concentration of 1 per cent erythrocytes in a final volume of 2 cc. of diluted toxin was reached. The degree of hemolysis was determined after one hour's incubation at 37 C. and after standing overnight at room temperature (or after overnight refrigeration when β toxin was used). To determine the degree of hemolysis, the tubes were centrifuged and compared

* Veal infusion broth, 1,000 cc., to which were added proteose peptone 1.0 per cent, KH_2PO_4 0.4 per cent, and $MgSO_4$ 0.06 per cent, with the resultant pH adjusted to 7.0.



Graph 1—The effect of inhibitory substances and β hemolysin on sheep erythrocytes.

to known standard** preparations of hemolyzed cells.

The laboratory unit of hemolytic toxin was selected as the smallest amount of toxin capable of producing 50 per cent hemolysis in 2 cc. of a 1 per cent erythrocyte suspension (*i.e.* h.d.$_{50}$).

The inhibitory substance was then titrated against 1 laboratory unit of hemolytic toxin. One cubic centimeter of saline dilutions of inhibitory substance were made and 1 laboratory unit of hemolytic toxin was added to each dilution; these agents were then incubated together for at least one hour prior to adding the red cells. This technique was altered (graphs 1 and 2) by adding the red cells to the dilutions of the inhibitory substance and incubating these together for one hour prior to adding the hemolytic toxin. After all ingredients were finally added, the tubes were incubated for an additional hour at 37 C. and the degree of hemolysis determined after standing overnight at room temperature (or after overnight refrigeration when β toxin was used). Hemolytic toxin, saline, and inhibitory substance controls were used.

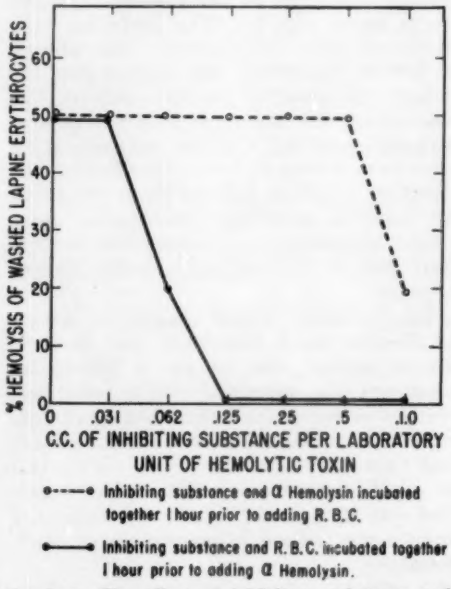
In order to determine whether the inhibitory substance was heat labile, tubes of the substance were held at 55 C. and 75 C. for thirty minutes and then titrated against 1 laboratory unit of β hemolysin (graph 3).

DISCUSSION

The action of the inhibitory substance against the α and β hemolysis *per se* is negligible or absent entirely (graphs 1 and 2). However, if the inhibitory substance is given the opportunity of acting on the red cells for a period of time prior to introducing the hemolysin, the lysis of the erythrocytes is prevented when critical quantities of the inhibiting substance are used.

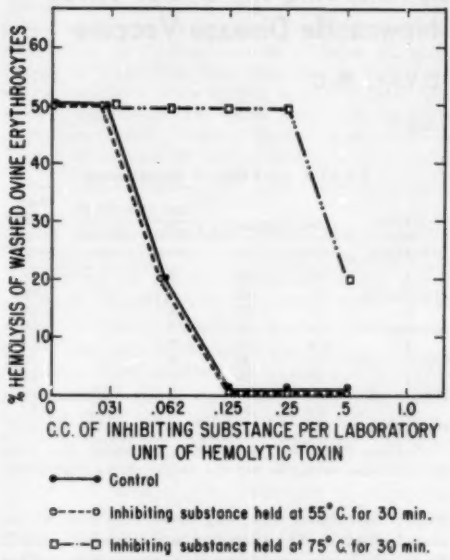
It is apparent from the results presented (graph 3) that the inhibitory substance is heat labile at temperatures of 75 C. acting for thirty minutes or more. Subjecting the inhibitory substance to 55 C. for thirty minutes did not alter its action sufficiently to be detected by these methods. The results shown in graph 3 were obtained by treating the red cells with the various samples of inhibitory substance prior to adding the hemolysin.

Addition of the inhibitory substance to plates of blood agar and subsequent inoculation of these plates with β hemolytic *Streptococcus agalactiae*, α, β, and α-β he-



Graph 2—The effect of inhibitory substance and α hemolysin on rabbit erythrocytes.

** These standards were prepared by adding from 0.5 cc. to 0.05 cc. of 4 per cent erythrocyte suspension to ten tubes, each tube receiving 0.05 cc. less than the preceding one. The final volume in each tube was brought to 2.0 cc. with distilled water. The erythrocytes used for the standards were from the same source as those used in the titrations of hemolysin.



Graph 3—The effect of temperature on the inhibitory substance activity with sheep erythrocytes and β hemolysin.

molysin-producing *M. pyogenes* prevented the occurrence of hemolysis about any of the resulting colonies. In addition, the reaction of Christie *et al.*,[2] the exaggerated zone of hemolysis which occurs where colonies of *Str. agalactiae* and β hemolysin-producing staphylococci grow in close proximity, was not evident in these plates which contained the inhibitory substance. It appears then that the substance is not specific for any one hemolysin but rather makes the red cell membrane more resistant to lysis.

The only other report of interference with hemolysis of this nature that has come to our attention was one by Munch-Petersen [3] in which he described a Corynebacterium capable of protecting erythrocytes against staphylococcal β toxin. Since we learned of his work after much of the work in this report was completed, comparisons were made to detect similarities or differences in the actions of the agents involved.

Munch-Petersen theorizes that his corynebacterium agent acts on the red cell membrane and thus prohibits lysis. This theory is advanced due to the absorption of the corynebacterial substance by the red cells and the resultant failure of these cells to lyse after the addition of staphylococcal β toxin. He also cites certain changes in red cell morphology which occur in cells treated with the agent.

The micrococcal inhibitory agent reported in this paper is apparently quite similar to Munch-Petersen's corynebacterial agent. This inhibitory substance also is probably active on the red cell membrane; however, its activity is lost after holding at temperatures in excess of 55 C., while Munch-Petersen's agent is no longer active after holding at 55 C.

Summary and Conclusions

A nonhemolytic Micrococcus is reported that elaborates a substance which is capable of preventing the lysis of red cells which are first treated with the inhibitory substance and then exposed to various hemolytic toxins.

The inhibitory substance apparently acts on the red cell membrane and makes it more resistant to lysis.

The inhibitory substance is heat labile.

The Micrococcus which elaborates this substance was isolated from a milk sample collected aseptically from a bovine mammary gland.

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The Immune Response in Chickens Following the Use of Three Different Types of Inactivated Newcastle Disease Vaccine

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THE PURPOSE of this investigation was to compare the immunity to challenge induced in chickens by three basic types of inactivated Newcastle disease vaccine.

PROCEDURE

Five experimental groups of chickens were made from 431 pullets obtained from a cross of two inbred lines of White Leghorn chickens. There were 20 unvaccinated controls and approximately 60 vaccinated chickens in each of the five groups. The initial vaccination of the chicks was delayed until they were 5 weeks of age because they were hatched from eggs laid by vaccinated stock. All vaccinated birds were given a 1-ml. dose of vaccine at 5 weeks of age. A portion of these was given a second 1-ml. dose three months later. The birds vaccinated once were challenged four months after vaccination while the birds vaccinated twice were challenged four months after revaccination.

The three types of vaccine were as follows: (*a*) two commercial, formalin-inactivated vaccines containing alumina-gel as an adjuvant; (*b*) two mineral oil vaccines represented by one commercial vaccine, inactivated by ultraviolet irradiation, and one experimental product in which the virus (Manhattan strain) was inactivated by 0.25 per cent formalin and the vaccine emulsified in a mineral oil according to Salk's[1] modification of the procedure of Freund *et al.*;[2] (*c*) one experimental crystal violet vaccine made by D. H. Legenhausen of the Animal Disease and Parasite Branch of the Agricultural Research Service at Beltsville, Md. The vaccine was dated Oct. 21, 1953, and was called No. 1. The GB-Texas-1948 * strain of virus was employed and the vaccine prepared according to the procedure of Doyle and Wright[3] in which a suspension of infected egg contents, ethylene glycol, and crystal violet is incubated at 37 C. for thirty-six days. All the vaccine, except the commercial mineral oil vaccine, was administered intramuscularly in the thigh. The commercial mineral oil vaccine was administered subcutaneously in the neck region.

The challenge strain of Newcastle disease virus (NDV) was GB-Texas-1948. The inoculum consisted of pooled allantoic fluid from embryos previously inoculated with lung suspension from an infected chicken. The inoculum was stored at −25 C. Each challenge inoculum was titrated in either embryos or chickens. The titer in chickens (tables 2 and 3) is the infective titer rather than the chicken lethal titer. Infection was determined by isolation of the virus from the blood or lung at ninety-six hours following inoculation.

TABLE 1—Plan of Experiment

Group and vaccine	No. chickens		Age (weeks) at		Age (weeks) at challenge
	Unvac.	Vac.	First vac.	Second vac.	
1	10	26	5		21
Alumina-gel	10	40	5	17	33
2	10	26	5		21
Alumina-gel	10	40	5	17	33
3	9	27	5		21
Crystal violet	10	39	5	17	33
4	10	29	5		21
Mineral oil	10	38	5	17	33
5	10	29	5		21
Mineral oil	10	38	5	17	33

Numbers given include 31 chickens which died from miscellaneous causes during the course of the experiment.

Resistance or susceptibillity to NDV was based on the presence or absence of virus in the blood or lung at ninety-six hours following chanllenge. In all but one instance, following aerosol challenge of the birds vaccinated once, blood was collected and the chickens killed at ninety-six hours for collection of lung tissue. The heparinized blood was placed in the refrigerator until the next day, centrifuged and the cells resuspended in broth prior to embryo inoculation. Lung was used in virus isolation attempts only when the blood sample was negative. If both blood and lung were negative for NDV the bird was considered resistant to the challenge dose administered.

Three variations in dosage and route of inoculation were used. Aerosol challenge was accomplished by placing 5 birds in each of six closed cages and exposing them to an aerosol produced by 50 strokes from a hand-pumped atomizer containing undiluted infected allantoic fluid. The birds were left in the cages ten minutes before the fan was turned on to permit a flow of air through the cages. Titration of the virus pool by aerosol inoculation was not done at the time of challenge, but a titration of the pool two months later using the same cages and the some technique in 4-month-old cockerels resulted in a titer of $10^{-4.5}$. The birds inoculated intratracheally were given a 0.1-ml. dose of a 1:100 dilution of infected allantoic fluid. The chickens inoculated intranasally were given 0.1 ml. of a 1:10 dilution of

From the Veterinary Medical Research Institute and the Animal Pathology Section of the Agricultural Experiment Station, Iowa State College, Ames. The author expresses appreciation for the cooperation given by the staff of the Veterinary Medical Research Institute, and to Miss Francais Weddle for technical assistance.

* This strain is highly pathogenic causing respiratory and paralytic symptoms and a high mortality in chickens.

infected allantoic fluid. Blood samples were collected on the day of challenge from 9 birds from each group of chickens vaccinated twice for serological testing. The hemagglutination inhibition (HI) tests were done by the *beta* procedure previously described.[4] The virus neutralization tests were done in embryonating chicken eggs using undiluted serum against tenfold dilutions of NDV.

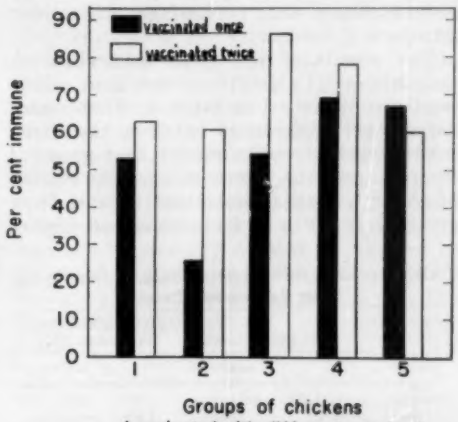
RESULTS

The results of challenge of the chickens vaccinated once are presented in table 2. The group of chickens challenged by aerosol were permitted to live after blood samples for virus isolation were collected at ninety-six hours. None of the 40 vaccinated chickens died or became paralyzed but the majority of them developed tracheal râles by the third day, an indication of respiratory infection. All 14 controls became paralyzed or died. The figures given in table 2 represent the number of infected birds based on virus isolation attempts from the blood at ninety-six hours.

The birds vaccinated once challenged either by intratracheal or intranasal inoculation were bled, killed, and lung tissue collected at ninety-six hours following challenge. The results (table 2) indicate the number of infected birds following challenge as determined by virus isolation from the blood or lung. These groups had no respiratory signs up to the time the birds were killed.

The challenge results (table 2 and graph 1) indicate that a greater percentage of chickens vaccinated once with the mineral oil vaccines resisted the NDV challenge than did birds vaccinated with the other two types of vaccine. However, based on *chi*-square [5] values this difference is not significant. The percentage of birds in group 2 (alumina-gel vaccine) which resisted challenge was significantly lower than for the other groups.

The results of challenging the chickens



Graph 1—Percentage of vaccinated chickens resistant to challenge with Newcastle disease virus.

vaccinated twice are presented in table 3. As indicated, three different challenge procedures were carried out. These figures are based on virus isolation attempts from the blood and lung at ninety-six hours following challenge. The majority of chickens challenged by aerosol had respiratory signs indicating infection when killed at ninety-six hours.

The challenge results of the chickens vaccinated twice (table 3, graph 1, and graph 2) indicate that a greater percentage of group 3 (crystal violet vaccine) and group 4 (mineral oil, ultraviolet-inactivated vaccine) chickens resisted the NDV

TABLE 2—Results of Challenging Chickens Vaccinated Once and Unvaccinated Control Chickens

Challenge route and virus dilution	Groups and type of vaccine										Titer** of challenge virus in	
	1 Alumina-gel		2 Alumina-gel		3 Crystal-violet		4 Mineral oil		5 Mineral oil			
	Vac.	Con.	Vac.	Con.	Vac.	Con.	Vac.	Con.	Vac.	Con.	Embryos	Chickens (age)
Aerosol undiluted	7/8*	2/2	8/8	3/3	5/8	3/3	2/8	3/3	2/8	3/3	$10^{-9.0}$	Not done
Intratracheal 1:100	1/11	4/4	4/10	4/4	3/8	3/3	2/10	4/4	3/9	4/4	$10^{-9.3}$	$10^{-7.3}$ 4 mo. male
Intranasal 1:10	4/7	3/3	7/8	3/3	3/8	3/3	4/8	3/3	3/7	3/3	$10^{-9.0}$	$10^{-8.7}$ 5 mo. pullets
Totals	12/26	9/9	19/26	10/10	11/24	9/9	8/26	10/10	8/24	10/10		

* Chickens infected by challenge/No. challenged; ** infective titer.

challenge than did the other groups. Based on *chi*-square values there was no significant difference between group 3 and group 4. The percentage of resistant birds in group 3, but not group 4, was significantly greater than the percentage given for groups 1, 2, and 5.

The results of the virus neutralization and hemagglutination-inhibition (HI) tests are presented in table 4. There were only slight differences between the virus neutralization results except that group 2 samples gave the lowest mean. The results of the HI tests indicate that serums from group 3 and group 4 chickens had higher titers of HI antibodies than samples from chickens in the other groups. Samples from group 2 chickens had the lowest titer of HI antibodies.

TABLE 4—Results of Testing Serums from Chickens Vaccinated Twice

Group and vaccine type	Virus neutralization: Neutralizing doses	Virus neutralization: Logarithm of geometric mean	Hemagglutination-inhibition (HI): HI units	Hemagglutination-inhibition (HI): Logarithm of geometric mean
1 Alumina gel	$1–10^4–10^{5*}$ $1–10^5–10^6$ $2–10^6–10^7$ $5–10^7–10^8$	6.4	1–200** 2–400 3–800 2–1600 1–3200	2.90
2 Alumina gel	$2–10^4–10^5$ $3–10^5–10^6$ $4–10^6–10^7$ $0–10^7–10^8$	5.6	1–100 3–200 2–400 3–800	2.59
3 Crystal violet	$0–10^4–10^5$ $1–10^5–10^6$ $5–10^6–10^7$ $3–10^7–10^8$	6.8	1–800 2–1600 4–3200 1–6400 1–12800	3.47
4 Mineral oil	$0–10^4–10^5$ $1–10^5–10^6$ $7–10^6–10^7$ $1–10^7–10^8$	6.4	2–800 2–1600 5–3200	3.30
5 Mineral oil	$0–10^4–10^5$ $2–10^5–10^6$ $6–10^6–10^7$ $1–10^7–10^8$	6.3	2–400 2–800 5–1600	3.00

* 1 sample tested between 10,000 and 100,000 neutralizing doses.

** 1 sample tested 200 HI units (1 HI unit is amount that will inhibit 1 hemagglutinating unit).

DISCUSSION

The challenge dose was varied in this experiment in order to obtain as much difference as possible in the results between the groups for comparison. As had been anticipated from previous challenge experiments, the birds vaccinated twice in all groups completely resisted a 0.1-ml. dose of a 1:100 dilution of infected allantoic fluid; however, when tenfold and one hundredfold amounts were given, differences in resistance between the groups became apparent (graph 2). Using the larger doses it was possible to differentiate between chickens with a high degree of resistance such as the chickens vaccinated twice with crystal violet vaccine and chickens with a lesser degree of resistance. This difference was apparent when either the 1:10 dilution or undiluted challenge material was used. The results obtained using the three different challenge doses emphasize the fact that it is possible to obtain a wide variation in the results depending upon the dosage, the route of inoculation, and the criteria used for recognizing infection following challenge.

The findings in the chickens once vaccinated confirm the results of previous experiments in which it was found that from 50 to 60 per cent of chickens vaccinated once at 4 and 8 weeks of age resisted the NDV challenge. The results with the chickens vaccinated once were not significantly different from the results with those vaccinated twice in groups 1, 4, and 5. However, there was a significant difference between the once-vaccinated and twice-vac-

TABLE 3—Results of Challenging Chickens Vaccinated Twice and Unvaccinated Control Chickens

Challenge route and virus dilution	Groups and type of vaccine: 1 Alumina-gel Vac.	1 Alumina-gel Con.	2 Alumina-gel Vac.	2 Alumina-gel Con.	3 Crystal violet Vac.	3 Crystal violet Con.	4 Mineral oil Vac.	4 Mineral oil Con.	5 Mineral oil Vac.	5 Mineral oil Con.	Titer** of challenge virus in: Embryos	Titer** of challenge virus in: Chickens (age)
Aerosol undiluted	7/10*	3/3	6/10	3/3	3/10	2/2	3/9	3/3	5/8	3/3	$10^{-8.5}$	Not done
Intratracheal 1:100	0/10	3/3	0/10	3/3	0/10	2/2	0/9	3/3	0/9	4/4	$10^{-8.7}$	Not done
Intranasal 1:10	7/18	3/3	9/18	3/3	2/17	3/3	4/15	4/4	9/18	3/3	$10^{-9.0}$	$10^{-8.4}$ 9 wk.
Totals	14/38	9/9	15/38	9/9	5/37	7/7	7/33	10/10	14/35	10/10		

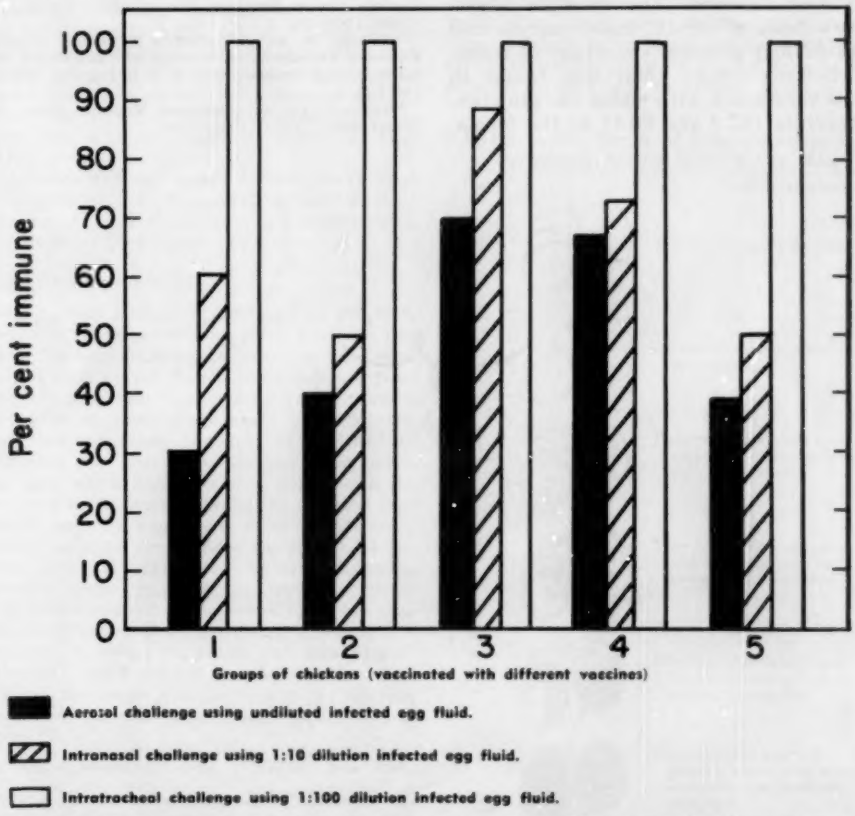
* Chickens infected by challenge/No. challenged; ** infective titer.

cinated chickens in groups 2 and 3. It is possible that the three-month interval between doses used in this experiment was not optimum for a significant anamnestic immune response in the chickens of groups 1, 4, and 5 when the initial dose of vaccine is given at 5 weeks of age.

The serum antibody levels as demonstrated with the HI test were higher than had been obtained previously for chickens vaccinated twice. However in previous experiments, the initial dose of vaccine had been given at an earlier age. That these antibody levels were due to the vaccines and not to adventitious infection or live virus in the vaccines was evidenced by the complete susceptibility of all controls. During the entire course of the experiment, the unvaccinated controls were kept in direct contact with those vaccinated.

The majority of virus isolations following challenge were made from the blood. The ability to isolate NDV from the blood of vaccinated birds following challenge is of interest, particularly since the virus neutralization tests indicated a high antibody content of the serums. A viremia existed in the vaccinated chickens in the presence of serum antibody. The virus apparently was associated with the cellular components of the blood since the samples were centrifuged and only the resuspended cells were used for embryo inoculation.

It is not possible to state what factors were responsible for the differences in the degree of immunity obtained from the inactivated vaccines used in this experiment because of the variation in strains of virus, inactivating procedures, and adjuvants used.



Graph 2—Percentage of twice-vaccinated chickens immune to different challenge doses of Newcastle disease virus.

SUMMARY

The degree of immunity induced among groups of chickens vaccinated with three different types of inactivated Newcastle disease vaccines was compared. The types of vaccines were: (*a*) two formalin-inactivated vaccines in which alumina-gel was used as the adjuvant; (*b*) a crystal violet vaccine in which ethylene glycol was incorporated; and (*c*) two vaccines in which mineral oil was the adjuvant used. One comparison was made following a single dose and another following two doses given three months apart. Challenge results of the once-vaccinated birds indicated no significant difference between the types of vaccine with the exception that the percentage of chickens resistant in the group vaccinated with one of the alumina-gel vaccines was significantly lower than for the other four vaccines. The chickens receiving two doses of crystal violet vaccine had a significantly greater percentage of resistant chickens (86.5) than was found in groups vaccinated with either the alumina-gel vaccines (63.2 and 60.5) or the formalin-inactivated, mineral oil vaccine (60.0). There was no significant difference, however, between the percentage of resistant birds in the group that received the crystal violet vaccine (86.5) and the group that received the ultraviolet-inactivated, mineral oil vaccine (78.8). The hemagglutination-inhibition antibody titer of the serums from chickens vaccinated twice in the latter two groups were greater than the antibody titer of serums from the other groups.

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The Effect of Piperazine-HCl on Migrating Larvae of *Ascaris Suum* Goeze 1782

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RECENT studies have demonstrated the vermifugal activity of several piperazine compounds against *Ascaris suum* Goeze 1782 of swine.

Leiper [2] treated 38 swine infected with ascaris with polymeric piperazine-1-carbodithioic acid and reports an egg count reduction of 96 per cent. Immature as well as adult worms were removed. Sloan *et al.*[1] have demonstrated the efficacy of piperazine adipate as a veterinary anthelmintic.

Although heavy infection with the adult worms interferes with the growth of young pigs, the larval migration through the lungs may also cause varying amounts of damage through hemorrhage and pneumonia. We were interested therefore to ascertain (1) whether piperazine will kill the ascaris larvae as they hatch out of the egg in the intestine and thereby prevent the lung migration, (2) the effect of piperazine on the migratory larvae in the blood stream and pulmonary system, and (3) the effect of piperazine administered to mother mice upon larval ascaris infection in their suckling young.

Piperazine-HCl was chosen for this study as it was demonstrated by Brown, Chan, and Hussey [5] to be one of the most effective anthelmintics of a large series of piperazines which they tested.

MATERIALS AND METHODS

Young, white mice weighing 17 to 22 Gm. from a colony free of nematodes were used in this study. The eggs employed to infect the mice were obtained from gravid *Ascaris suum* obtained from swine, and they were incubated for twelve to fourteen weeks at room temperature until the embryos were completely developed. Like other investigators, we began our feeding experiments with eggs which had developed active embryos after four to six weeks' incubation at room temperature and the mice were never satisfactorily infected, as most of the eggs passed out of the host's intestine unhatched. Larsh,[3] employing eggs in a similar stage of development, reported on the relationship of the intestinal emptying time in mice and their natural resistance to pig ascaris infections. However, we found that mice in general showed less resistance to infection when eggs incubated for longer periods were used. Between experiments, the embryonated eggs were stored in a refrigerator at 0 to 4 C. Each animal used in the study was given 5,000 ascaris eggs by stomach tube and the suckling mice were given 3,000.

To test the efficacy of piperazine against the hatching larvae, a dose of piperazine was given one half to one hour before the administration of the embryonated eggs, and thereafter at hourly intervals for a total of six doses. The drug was administered by stomach tube and the total dosage of piperazine-HCl employed was 300 mg. or 600 mg. per kilogram body weight of the mice. The mice were necropsied seven or ten days after the completion of the piperazine therapy. Ascaris-infected but untreated mice served as controls.

To ascertain the effect of piperazine on the migrating ascaris larvae, chemotherapy was initiated twenty-four hours after feeding the infectious eggs. A single daily dose of 500 mg. per kilogram of piperazine was given by stomach tube on seven consecutive days, and to another group of mice

TABLE 1—The Degree of Lung Pathology in Experimental Ascaris Infections in Mice

Class	Gross lung lesions	
0		Lungs apparently normal.
1		Presence of from 1 to 6 petechial spots.
2		Presence of scattered petechial spots with a few confluent ecchymotic areas.
3		Presence of petechial spots and confluent ecchymotic areas of moderate size.
4		Presence of petechial spots and confluent ecchymotic areas of larger size.
5		Appearance of complete hepatization of both lungs, or animal died from the infection.

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the drug was given intraperitoneally for three or seven days. The mice were necropsied thirteen days after infection or five days after the completion of therapy.

At necropsy, the liver and lungs of both the treated mice and untreated control mice were examined for the presence of larvae by the tissue compression technique. The gross pathological pulmonary lesions were graded from 1 to 5, according to the extent of their consolidation (table 1). This classification made it possible to compare the pulmonary damage in groups of mice.

TABLE 2—Effect of Oral Piperazine-HCl on Ascaris Larvae in Mice Necropsied Seven Days after Infection

Mouse No.	Gross lung lesions: Treated* mice 100 mg./kg. x 6	Gross lung lesions: Control mice
1	0	4
2	0	4
3	0	5
4	1	3
5	0	3
Av.	0.20	3.8

* Drug given orally thirty to sixty minutes before administration of infective eggs and for five hours afterward.

Results

The mice which were given piperazine thirty to sixty minutes before administration of infective ascaris eggs, and for five hours thereafter, passed a large number of ascaris larvae in their fecal pellets. Signs of pneumonia did not appear in any of the treated animals and necropsy revealed only minimal pulmonary lesions in 5 of the 19 mice with few or no larvae in the lung tissues (tables 2 and 3). The untreated groups of mice, however, passed relatively few larvae in their stools and there were three deaths among the group due to pneumonia. On necropsy all of this control group of mice exhibited extensive pulmonary consolidation. It is apparent from these experiments that the ascaris larvae in the intestine of the mice were affected sufficiently by the piperazine-HCl to prevent their normal blood stream and pulmonary migration and, instead, many of the larvae were passed in the feces of the mice. Six consecutive hourly doses at the rate of 50 mg./kg. and 100 mg./kg. both appeared to be highly effective against the larvae.

TABLE 3—Effect of Oral Piperazine-HCl on Ascaris Larvae in Mice Necropsied Ten Days after Infection

Mouse No.	Gross lung lesions: Treated* groups 50 mg./kg. x 6	Gross lung lesions: Treated* groups 100 mg./kg. x 6	Gross lung lesions: Control group
1	0	2	3
2	1	0	4
3	1	0	4
4	0	2	4
5	0	0	5 (died)
6	0	0	5 (died)
7	0	0	5 (died)
Av.	0.29	0.57	4.3

* Drug given orally thirty to sixty minutes before administration of infective eggs and for five hours afterward.

The effect of piperazine-HCl upon larvae already in the migratory phase of their life cycle is shown in table 4. The experimental mice were given 5,000 infective ascaris eggs each and twenty-four hours later drug therapy was commenced and continued for seven consecutive days. Both the treated mice and the untreated control animals exhibited pneumonia, and mice in each group died of the infection. At necropsy, thirteen days later, there was evidence of healing of the pulmonary lesions in the surviving animals. This experiment suggests that piperazine-HCl in the dosage employed did not reach the blood stream and tissues in sufficient concentration to prevent the pulmonary migration of the ascaris larvae.

TABLE 4—Effect of Oral Piperazine-HCl on Migrating Ascaris Larvae in Mice Necropsied Thirteen Days after Infection

Mouse No.	Gross lung lesions: Treated* Group 500 mg./kg. x 7	Gross lung lesions: Control group
1	2	2
2	3	1
3	2	2
4	2	2
5	2	1
6	2	1
7	2	5 (died)
8	2	5 (died)
9	5 (died)	5 (died)
10	5 (died)	5 (died)
Av.	2.7	2.9

* Drug given orally twenty-four hours after administration of infective eggs and six consecutive days thereafter.

The failure of piperazine-HCl administered orally to have a noticeable effect upon migrating larvae is probably due to a low blood and tissue concentration.[4] In an effort to overcome this, groups of mice were given the piperazine-HCl intraperitoneally for three to seven days beginning twenty-four hours after the admin-

istration of the embryonated ascaris eggs (table 5). Necropsy of the treated groups of mice and untreated control mice ten

TABLE 5—Effect of Piperazine-HCl Given Intraperitoneally upon Ascaris Larvae in Mice Necropsied Ten Days after Infection

Mouse No.	Gross lung lesions			
	Treated* mice 300 mg./kg. x 3	Control mice	Treated* mice 300 mg./kg. x 7	Control mice
1	1	3	2	3
2	3	2	1	5
3	2	4	3	4
4	3	4	3	3
5	2	5	2	3
6	2	3	3	5 (died)
7	1	3	2	5 (died)
8	3	3	....	....
9	2	3	....	....
10	2	4	....	....
Av.	2.1	3.4	2.3	4.0

* Drug given twenty-four hours after administration of infective eggs and continued for three to seven days.

days after infection revealed slightly less pulmonary hemorrhage and consolidation in the former groups than the latter. It appears possible that sufficient drug reached the blood-borne larvae to prevent the migration of some of them out of the blood stream into the pulmonary tissues.

TABLE 6—The Course of Ascaris Pneumonia in Suckling Mice Whose Mother Was Orally Administered Piperazine-HCl Three Hours before and after Their Ascaris Infection — Mice Necropsied Eight Days after Infection

Suckling mouse No.	Gross lung lesions in suckling mice	
	Mother treated 500 mg./kg. x 3	Mother untreated control
1	4	1
2	2	2
3	3	1
4	2	3
5	4	3
6	0	4
7	4	1
Av.	2.7	2.1

A single experiment was conducted on the effect of piperazine-HCl in mother's milk upon the newly hatched ascaris larvae in the intestine of suckling young. A mother mouse was given orally 500 mg./kg. of piperazine-HCl three hours before her suckling young were each given 3,000 infective ascaris eggs. Additional doses of the drug were administered to the mother at the time the young received the eggs and also three hours later, a total of 3 doses over a period of six hours. A control litter of mice was similarly fed ascaris eggs and they continued to suckle their mother which was not given piperazine-HCl. The results (table 6) indicate that insufficient drug (if any) was secreted in the milk to affect the newly hatched larvae in the intestine of the young mice. The young of both the treated and untreated mother exhibited extensive pulmonary consolidation.

SUMMARY

Piperazine-HCl administered orally to mice before and after feeding them infective *Ascaris suum* eggs prevented the hatching larvae from invading the blood stream and the lungs. The drug fed after invasion of the blood stream by the larvae did not prevent their invasion into the lungs and a severe pneumonia. Intraperitoneal injections of piperazine-HCl had slight effect on the migrating larvae. There was no evidence that the drug, in the amount employed, was excreted in the milk in sufficient quantities to effect the newly hatched larvae in the intestine of suckling mice.

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The pH Stability of the Wisconsin FXNO Strain of Egg-Adapted Distemper Virus

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THE VIABILITY of distemper virus (DV) at various hydrogen ion concentrations is of fundamental importance in the study of its characteristics.

Bindrich[1] in 1951 studied this problem using blood serum from infected dogs as a virus source. He mixed serum containing DV with phosphate buffer solutions of various pH values and injected this mixture into susceptible dogs. By observance of clinical signs, he concluded that distemper virus was resistant to acid, "weakened" at pH 9 and completely inactivated in higher alkaline concentrations. Celiker and Gillespie[2] recently reported on the survival of distemper virus *in ovo*. They used various buffers and obtained survival for twenty-four hours between the pH values of 4.4 and 10.4. Beyond these values, no viability could be demonstrated. These results were reported on an "all or none" basis indicating either survival or nonsurvival of virus at a designated pH.

Since it was probable that differences in percentage of viability existed within the range reported, the present investigation was conducted in an attempt to demonstrate the percentage viable at various hydrogen ion concentrations.

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MATERIALS AND METHODS

Preparation of Stock Virus.—The virus suspensions for testing were prepared from infected chorioallantoic membranes (c.a.m.) of the seventieth passage level of the Wisconsin FXNO strain of egg-adapted DV.[3] Seven-day-old chicken embryos were inoculated on the c.a.m. with 0.1 ml. of seed virus. These embryos were further incubated for six days and the infected membranes

TABLE 1—Survival of Distemper Virus* in Veronal-Acetate Buffers

Exp. No.	Exposure to buffer before inoculation	pH	Replicate† 1	2	3	4	Total	Per cent survival total
1	12 hr. at 5 C.	2.82	2/12	2/12	3/12	0/12	7/48	14.6
		3.62	3/12	4/12	7/11	0/12	14/47	29.8
		4.92	4/12	3/12	4/12	3/11	14/47	29.8
		5.42	1/9	3/12	4/11	6/10	14/42	33.3
		6.48	6/11	1/12	8/12	6/9	21/44	48.8
		7.63	6/11	4/12	3/11	8/9	21/43	49.0
		8.82	9/12	3/11	9/11	6/7	27/41	65.9
		9.28	10/11	7/12	9/12	2/6	28/41	68.3
	Undiluted virus control		16/16	10/11	11/11	8/8	45/46	97.8
2	24 hr. at 5 C.	2.82	0/12	3/12	1/12	2/11	6/47	12.8
		3.62	1/12	2/11	3/12	2/9	8/44	18.2
		4.92	2/9	3/8	5/11	5/8	15/36	41.7
		5.42	6/10	4/5	6/6	7/8	23/29	79.3
		6.48	6/9	8/8	5/6	6/7	25/30	83.5
		7.63	8/8	8/8	7/7	8/9	31/32	97.0
		8.82	3/3	7/7	9/9	9/9	28/28	100.0
		9.28	3/4	6/8	9/10	8/8	26/30	86.7
	Undiluted virus control		8/11	7/7	9/9	6/6	30/33	90.9
3	30 min. at 37 C.	2.82	.....	2/12	1/9	.....	3/21	14.3
		3.62	2/11	5/10	5/9	.....	12/30	40.0
		4.92	4/11	2/12	4/8	.....	10/31	32.3
		5.42	3/11	4/12	4/9	.....	11/32	34.4
		6.48	2/11	4/10	5/9	.....	11/30	36.7
		7.63	5/12	4/13	6/9	.....	15/34	44.1
		8.82	8/12	.....	4/9	.....	12/21	57.1
		9.28	5/11	3/10	3/9	.....	11/30	36.7
	Undiluted virus control		4/6	12/12	12/12	.....	28/30	90.3

* Wisconsin FXNO strain; 50 per cent infective dose ($i.d._{50}$) = $10^{4.4}$ when 0.1 ml. of inoculum is injected into chicken embryos.

† Numerator = number of eggs displaying lesions; denominator = total number of eggs injected and living at the time of harvest.

were harvested, weighed, and combined with a diluent of 10 per cent beef infusion broth in saline to make a final dilution of 10^{-1}. To each milliliter of suspension was added 400 units of penicillin G and 40 μg. of streptomycin dihydrochloride. This mixture was centrifuged at 6,000 r.p.m. at 2 C. for thirty minutes. The supernatant fluids were removed, mixed, and distributed in 4-ml. quantities in rubber-stoppered ampules. Following preparation of virus, the 50 per cent infective dose in chicken embryos ($i.d._{50}$) was determined. These ampules were stored at −20 C. The stock virus was prepared at monthly intervals.

Buffer Solutions.—The buffer employed was veronal-acetate prepared according to the method of Michaelis.[4] The use of one type of buffer for the entire range studied eliminated possible differences in effect due to different buffer components. Boiled distilled water was used in the preparation of the buffer solutions. The pH of the buffer solutions and the buffer-virus mixtures was determined with a model "G" Beckman pH meter at the time of each test. When streaked on blood agar plates, the mixtures gave no growth.

Experimental Procedure.—To 4.5 ml. of buffer solution, 0.5 ml. of a 10^{-3} dilution of virus, thawed at room temperature, was added resulting in a final virus dilution of 10^{-4}. This buffer-virus mixture (0.1 ml.) was inoculated onto the chorioallantoic membranes of 7-day-old embryos and examined following six days of incubation. The membranes were observed for distemper virus plaques, three or more plaques being considered evidence of viability. Dead eggs were eliminated from the calculations.

Experiments.—Three experiments were performed; four replicates were conducted on the first two and three replicates on the third experiment. The trials consisted of (1) virus-buffer mixture incubated for twelve hours at 5 C. before inoculation, (2) virus-buffer mixture incubated for twenty-four hours at 5 C. before inoculation, and (3) virus-buffer mixture incubated for thirty minutes at 37 C. before inoculation.

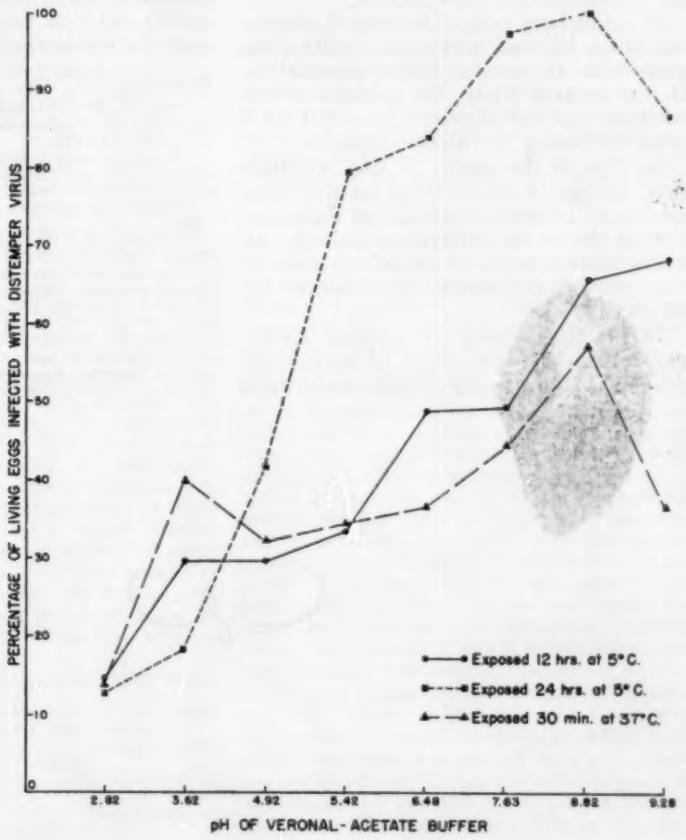

Fig. 1—The pH stability of Wisconsin FXNO strain of egg-adapted distemper virus.

RESULTS AND DISCUSSION

In these experiments (table 1 and fig. 1), the distemper virus was more resistant to alkaline pH than to acid; this was consistent in all three experiments, though it can be seen most readily in the experiment where the mixture was held at 5 C. for twenty-four hours. The reason for the increased tolerance to pH in this experiment is not readily discernible. Virus activity appears to decline at about pH 9.28. It was not possible to exceed this pH with a single buffer. These results are not in complete agreement with those of Bindrich [1] who recommended around pH 7.0 as optimal; on the basis of our results, the optimal pH is higher. As can be seen from the results in table 1, there was great variation between replicates; however, the individual replicates were generally more resistant to alkaline pH than to acid.

It would have been of interest to observe the effect of readjusting the buffer-virus suspension to neutral before inoculation. In the present study, the reaction of the solution was not returned to a pH of 7 prior to testing in chicken embryos.

In view of the results of this investigation, the use of lye solution for the disinfection of infected materials in distemper control should be critically examined. Alkaline disinfectants, if excessively diluted, may provide a favorable environment for survival.

In the later stages of catarrhal distemper and early in the course of neurotropic distemper, the urinary bladder epithelium often contains numerous inclusion bodies. That these bodies contain viral antigen has recently been demonstrated by Moulton and Brown [5] and by Coffin.[6] Gorham and Brandly [7] isolated virulent virus from bladder tissue but not from urine. The results of this investigation indicate that an egg-shaped strain of DV is susceptible to acid pH. Inasmuch as dog and mink urine is usually acid there would be a possibility of virulent virus being inactivated in urine.

SUMMARY

1) The pH stability of the Wisconsin FXNO strain of distemper virus incubated with Michaelis veronal-acetate buffers ranging from pH 2.82 to pH 9.28 was investigated.

2) This strain is more stable at alkaline pH than at acid; these results are suggestive of greater virus stability at approximately pH 8.82 than at alkaline reactions immediately above or below.

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Studies on the Preservation of *Leptospira Icterohaemorrhagiae* by Freezing

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PRESERVATION of viability and virulence of the pathogenic spirochetes has long been a problem to those working with these organisms and as yet no completely satisfactory method has been reported. The present study was undertaken in an attempt to learn if suitable combinations of suspending menstruum and storage temperature could be employed for the successful preservation of *Leptospira icterohaemorrhagiae*. The choice of this spirochete as the test organism was prompted by the fact that among the pathogenic spirochetes, members of the genus Leptospira are the easiest to cultivate *in vitro*. Also, members of this genus are the cause of leptospirosis in man and animals, a disease that has assumed increasing importance in recent years.

LITERATURE CITED

There are a number of factors which appear to have an effect on the susceptibility of microorganisms to freezing. Among these are the temperature of the freezing mixture, the storage period while frozen, abruptness of the temperature changes and the nature of the suspending menstruum. To study the effect of the temperature of the freezing mixture, Hilliard *et al.*[1] froze and held cultures for three hours at temperatures of approximately —15 and —2 C. It was noted that the lower temperature had a greater effect on the reduction of the number of viable organisms. In contrast, Haines[2] observed that the temperature or the rate of freezing had little effect on cell mortality. Studies of the effects of rapid freezings and thawings on 12 different bacterial cultures by Smith and Swingle[3] showed a gradual reduction of the living population to zero. Hilliard *et al.*[1] stated that freezing and thawing at intervals was considerably more lethal than continuous freezing. In contrast, Tanner and Wallace[4] reported that alternate freezing and thawing was no more destructive to microorganisms than continuous freezing.

The findings of Haines[2] suggested some correlation between storage temperature and survival of microorganisms. When frozen aqueous suspensions of bacteria were stored at temperatures ranging from —1 to —20 C., the most rapid death rate occurred near the highest storage temperature.

The nature of the suspending menstruum appears to have an important bearing on how well microorganisms withstand the adverse effects of initial freezing and subsequent storage at low temperature. The formation of ice crystals, resulting in the mechanical disruption of cells, is thought to be one of the chief factors in the reduction of bacterial populations undergoing freezing. Those substances which are colloidal in nature appear to afford the greatest degree of protection against this disruption. Keith[5] compared the survival time of *Escherichia coli* in different suspending menstruums. One group of cultures was suspended in milk and another group in tap water and frozen. Milk afforded a greater degree of protection than did tap water. These findings were substantiated by Hilliard and Davis,[6] Prucha and Brannon,[7] and Tanner and Wallace.[4]

Studies on the preservation of spirochetes by freezing have been of relatively recent date and the number of reports in the literature few. Turner[8] reported preserving two species of treponemes, *Treponema pallidum* and *Treponema pertenue*, in the frozen state for as long as one year when maintained at a temperature of —78 C. As Haines[2] had noted with other bacteria, a difference was apparent in survival times of treponemes held at different temperatures. Those stored at —10 and —20 C. failed to survive for as long as two months. Death of the organisms occurred during the maintenance period and not at the time of the initial freezing. Turner and Fleming[9] reported on the viability and virulence of different spirochetes when stored at —78 C. for varying periods. *Treponema pallidum* and *T. pertenue* were actively motile and were highly pathogenic for rabbits after storage for approximately three years. Relapsing fever spirochetes, tested after storage for six months to one year, showed active motility and were virulent for mice. The spirochete of rat bite fever, *Spirillum minus*, was virulent for mice after storage for one year. *Leptospira icterohaemorrhagiae* was actively motile after storage for five, six, and ten months. Stavitsky[10] demonstrated viability and virulence of *L. icterohaemorrhagiae* for one hundred days when blocks from infected guinea pig livers were frozen and stored at —20 C. Ten per cent liver suspensions frozen and stored at —20 C. showed viability and virulence for only fifteen days and cultures

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frozen and stored showed viability and virulence for only five days.

MATERIALS AND METHODS

The culture of *L. icterohaemorrhagiae* used in this study was obtained from Dr. John P. Newman of the Department of Microbiology and Public Health, Michigan State University. It was designated as strain 871 and was originally isolated from a case of leptospirosis in man at the Walter Reed Army Hospital, Washington, D. C. The organism was grown in Korthoff's medium to which had been added 10 per cent sterile normal inactivated horse serum. The medium was tubed in 10-ml. amounts in screw-cap vials, and each vial was seeded with 0.25 ml. of actively motile, 7- to 9-day old culture. These were incubated at a temperature of approximately 30 C. for seven to nine days after which time the contents of five vials (50 ml.) were pooled. Dark-field examinations were made on these pooled lots to determine purity of culture and motility of the leptospiras. Only pure, actively motile cultures were processed further. The spirochetes were concentrated by centrifugation employing a refrigerated centrifuge * at a temperature of approximately 8 C. and a relative centrifugal force of approximately 1,350 x g. The supernatant fluid was discarded and the sediment resuspended in 5 ml. of the suspending menstruums employed. The substances used for suspension were: 1 and 2 per cent isoelectric casein (Difco), skim milk (Difco), 1 per cent starch, 1 and 3 per cent gelatin, 0.25 per cent agar, egg yolk, fresh allantoic fluid, allantoic fluid first frozen and then thawed and the precipitate removed and discarded (designated hereafter as frozen allantoic fluid), normal rabbit serum, and normal horse serum. With the exception of the serums, egg yolk, and allantoic fluids these substances were adjusted to a pH of 7.4 and sterilized by autoclaving at 121 C. for fifteen minutes. The serums were sterilized by passage through Hercules ST-3, seitz-type filter pads. Egg yolk was obtained by aseptically aspirating the yolk contents from 4- to 6-day-old embryonating chicken eggs. Allantoic fluid was obtained by aseptically aspirating the contents of the allantoic cavity of 12- to 13-day-old embryonating chicken eggs.

Following suspension of the spirochetes in each of the suspending menstruums, approximately 0.5-ml. amounts were placed in sterile 4-inch tubes made from 8-mm. pyrex tubing. These were plugged with sterile cotton and sealed with parafilm. A sample of each suspension was inoculated into Korthoff's medium for controls to verify the presence of viable organisms in the original suspension. These were incubated as were the original seed cultures. The remaining samples were sharp-frozen by immersion for five to ten minutes in a dry ice-alcohol bath at approximately —70 C. One sample of each frozen suspension was allowed to thaw at room temperature and when thawed was inoculated into Korthoff's medium and incubated. This was to determine if the organisms had survived the original freezing process. The remaining frozen specimens were placed in suitable containers and stored in freezing units at different subzero temperatures. The storage units employed were: (1) a commercial cold hold unit maintaining a temperature of approximately —47 C.; (2) a revco unit maintaining a temperature of approximately —27 C.; (3) a dry ice storage chest in which the temperature fluctuated from approximately —60 to —30 C. depending on the amount of ice in the chest and how frequently the chest was opened. Samples were removed from storage at designated intervals, allowed to thaw at room temperature and when liquefied were inoculated into Korthoff's medium and incubated. The cultures were incubated at 30 C. for two weeks after which time tubes were examined grossly by transmitted light for evidence of growth, and microscopically by means of dark field for purity and motility.

RESULTS

Results are shown in the accompanying tables. In all instances the original suspension, prior to freezing, contained viable leptospiras. Fresh allantoic fluid and 0.25 per cent agar were the only mentruums which did not consistently protect during the initial sharp freeze. Table 1 shows the

* International Equipment Co., model PR-1.

TABLE 1—Longevity of Leptospira Icterohaemorrhagiae Suspended in Various Menstruums and Stored at —47 C.

	Suspending menstruum										
Storage time	Starch (1%)	Gelatin (1%)	Gelatin (3%)	Agar (0.25%)	Casein (1%)	Casein (2%)	Skim milk	Egg yolk	Normal horse serum	Fresh allantoic fluid	Frozen allantoic fluid
Not frozen	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
Frozen and thawed immediately	4+	4+	4+	—	4+	3+	4+	4+	4+	3+	3+
One month	—	—	3+	—	+	—	3+	4+	3+	3+	—
Three months	—	+	—	—	2+	3+	4+	4+	—	—	3+
Six months	—	—	2+	—	2+	4+	4+	2+	4+	—	—
One year	—	—	3+	—	2+	3+	4+	3+	4+	—	—

Incubation at 30 C. for two weeks: — = no growth: +, 2+, 3+, 4+ = estimated amount of growth.

TABLE 2—Longevity of Leptospira Icterohaemorrhagiae Suspended in Various Menstruums and Stored at —27 C.

	Suspending menstruum										
Storage time	Starch (1%)	Gelatin (1%)	Gelatin (3%)	Agar (0.25%)	Casein (1%)	Casein (2%)	Skim milk	Egg yolk	Normal rabbit serum	Fresh allantoic fluid	Frozen allantoic fluid
Not frozen	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
Frozen and thawed immediately	3+	3+	2+	+	2+	3+	4+	+	4+	—	3+
One month	3+	—	3+	—	+	+	4+	3+	—	—	—
Three months	3+	3+	3+	3+	+	—	4+	—	+	—	—
Five months	2+	4+	4+	—	—	3+	4+	—	—	—	—
Nine months	4+	—	2+	—	—	4+	—	—	—	—	—
One year	4+	—	4+	—	—	—	4+	—	—	—	—

Incubation at 30 C. for two weeks; — = no growth; +, 2+, 3+, 4+ = estimated amount of growth.

results of storing suspensions at —47 C. After twelve months of storage, those menstruums containing viable leptospiras were: 3 per cent gelatin, 1 and 2 per cent casein, skim milk, normal horse serum, and egg yolk. These were likewise the only suspensions that showed evidence of viability at the end of six months of storage.

Table 2 shows the results of storing suspensions at —27 C. After nine to twelve months in storage, the menstruums supporting or protecting viability were: 1 per cent starch, 3 per cent gelatin, 2 per cent casein, and skim milk.

Table 3 shows the results of storing suspensions in a dry ice storage chest. After eight months' storage, the menstruums containing viable organisms were skim milk and normal horse serum.

In most instances, dark-field examination revealed leptospiras that were normal morphologically and actively motile. Some granulation was observed in those organisms which had been suspended in 1 per cent starch and in 1 and 2 per cent casein.

Discussion

Viability of *L. icterohaemorrhagiae* has been maintained for periods up to one year when sharp-frozen in suitable menstruums and stored at specified subzero temperatures. Of the menstruums tested, normal horse serum and skim milk appeared to be the most efficacious in protecting viability while 3 per cent gelatin and 2 per cent casein were slightly less effective. In the only experiment in which normal rabbit serum was used (table 2) failure of survival was noted after a three months' storage period at —27 C. In an unreported experiment, viability was demonstrated for nine months but not for twelve months when leptospiras were suspended in normal rabbit serum and stored at —47 C. It may be that normal horse serum is superior to normal rabbit serum as a suspending menstruum.

Virulence studies on *L. icterohaemorrhagiae* stored for one year at —47 C. to date are only preliminary. Half-grown hamsters were inoculated intraperitoneally with 0.5 ml. of 7-day-old subcultures made from organisms suspended in 3 per cent gelatin, 1 and 2 per cent casein, skim milk, and normal horse serum. Hamsters receiving the subcultures from leptospiras suspended in 3 per cent gelatin and 2 per cent casein died seven and nine days, respectively, after the injection. Hamsters receiving

TABLE 3—Longevity of Leptospira Icterohaemorrhagiae suspended in Various Menstruums and Stored in a Dry Ice Storage Chest

	Suspending menstruum										
Storage time	Starch (1%)	Gelatin (1%)	Gelatin (3%)	Agar (0.25%)	Casein (1%)	Casein (2%)	Skim milk	Egg yolk	Normal horse serum	Fresh allantoic fluid	Frozen allantoic fluid
Not frozen	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
Frozen and thawed immediately	2+	+	2+	—	3+	3+	4+	3+	3+	—	3+
One month	+	4+	4+	—	4+	4+	3+	4+	4+	4+	4+
Three months	3+	—	3+	—	3+	4+	4+	+	4+	—	3+
Four months	—	—	—	—	—	3+	4+	—	4+	—	—
Eight months	—	—	—	—	—	—	4+	—	3+	—	—

Incubation at 30 C. for two weeks; — = no growth; +, 2+, 3+, 4+ = estimated amount of growth.

subcultures from leptospiras suspended in 1 per cent casein, skim milk, and normal horse serum survived the inoculation. Further studies on this phase and continued studies on viability are in progress.

SUMMARY

Viability of *Leptospira icterohaemorrhagiae* grown in Korthoff's medium, concentrated by centrifugation, suspended in various colloidal substances, and stored at subzero temperatures has been demonstrated for periods up to one year. Leptospiras survived for at least eight months when suspended in skim milk or in normal horse serum and stored in a dry ice chest. When suspended in 1 per cent starch, 3 per cent gelatin, 2 per cent casein, and skim milk and stored at —27 C., viability has been demonstrated for nine to twelve months. When suspended in 3 per cent gelatin, 1 and 2 per cent casein, skim milk, normal horse serum, and egg yolk and stored at a temperature of —47 C., survival has been demonstrated for one year.

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Isolation of Vesicular Stomatitis Virus from an Infected Laboratory Worker

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DIAGNOSIS of human infections with vesicular stomatitis (VS) virus, based on serological findings, have been reviewed by Hanson *et al.*,[1] and additional similarly diagnosed cases have occurred at the Animal Disease Station, Beltsville, Maryland.[2] In none of these cases, however, was the virus isolated.

The virus of VS has been under investigation in cattle, swine, horses, sheep, guinea pigs, mice, embryonating chicken eggs, and tissue culture at the Plum Island Animal Disease Laboratory. A professional worker (FW) was exposed to the New Jersey type of the virus, most likely either while inoculating cattle in the tongue epithelium on July 20, 1954, or while examining infected cattle on July 21. On the latter date, it was observed that mucus from the mouth of a cow was thrown into his face and eyes. By the afternoon on July 22, the worker was feeling ill with chills. The man's wife, a nurse, made a record of the episode as summarized in table 1.

The temperature was normal after July 29, and there was gradual resolution of the throat lesions. Throughout the period of illness, the patient experienced malaise and depression. A blood sample was taken on July 23, part of which was citrated and the remainder allowed to clot. Nasopharyngeal washings with physiological saline were also obtained. The blood and nasopharyngeal washings were immediately refrigerated at home and brought to the Plum Island laboratory on July 26. The patient returned to work on August 2.

EXPERIMENTAL DATA

Preparation of Materials.—The patient's blood without anticoagulant was centrifuged, the serum removed and frozen in a flame-sealed ampule for future reference. The blood containing sodium citrate (20 mg./ml. of blood) and the nasopharyngeal washings were each prepared for use by adding 1,000 units of penicillin and 1 mg. of streptomycin per milliliter and placed at 4 C. for one hour.

The nasopharyngeal washings and citrated blood were inoculated into the allantoic cavity of 8-day embryonating chicken eggs and intracerabrally into 21-day-old mice, the doses being 0.2 ml. and 0.03 ml., respectively. No signs of infection were observed in the mice or embryonating chicken eggs inoculated with the nasopharyngeal washings. However, deaths of the embryos inoculated with blood occurred on the third day of incubation. The dead embryos were extremely hemorrhagic and friable. From one such embryo a second passage was made in 8-day embryonating chicken eggs by means of whole ground embryo diluted 10^{-2} with broth and preserved at —50 C. in flame-sealed ampules. A third passage was made in eggs to obtain a stock of the causative agent (FW agent) and to determine its l.d.$_{50}$ titer. A bacteria-free stock of the FW agent was obtained with a titer of $10^{-6.5}$ as determined by the method of Reed and Muench.[3] A fourth passage made in 7-day embryonating chicken eggs at a later date had an l.d.$_{50}$ titer of $10^{-9.0}$.

The dilutions of 10^{-2}, 10^{-3}, and 10^{-4} of the third egg passage material were inoculated into the rear plantar pads of 2 guinea pigs per dilution. All inoculated guinea pig pads developed vesicular lesions and material was harvested at forty-eight hours. A steer inoculated intradermally in multiple sites in the tongue with FW agent developed a temperature of 104.5 F. and vesicular lesions within twenty-four hours. Thirty grams of infected tongue tissue were harvested.

Virus Neutralization Experiments.—A virus neutralization test was conducted in 8-day embryonating chicken eggs involving the FW agent,

TABLE 1—Clinical Observations of Worker (FW)

Date	Time	Temperatures	Comments
July 22	2:30 p.m.	Not taken	General feeling of illness with chills
	5:30 p.m.	100.0	
	7:00 p.m.	102.0	
July 23	12:00 mid	102.0	.....
	7:00 a.m.	101.6	
	12:00 noon	101.4	
	6:00 p.m.	99.8	
July 24	7:00 a.m.	98.8	Felt normal
July 25			Sore throat with small, circumscribed flat yellow lesions.
July 26	12:00 noon	99.4	Physician called
	6:00 p.m.	101.4	and terramycin®
	10:00 p.m.	102.0	administered.
July 27	7:00 a.m.	102.0	.....
	12:00 noon	101.4	
	6:00 p.m.	101.4	
July 28	7:00 a.m.	99.6	.....
	12:00 noon	99.6	
	6:00 p.m.	99.4	
July 29	7:00 a.m.	98.8	.....
	12:00 noon	98.8	
	6:00 p.m.	98.8	

From the Plum Island Animal Disease Laboratory, Animal Disease and Parasite Research Branch, A.R.S., U. S. Department of Agriculture.

TABLE 2—Neutralization Test of the FW Agent in Eggs

Inoculum	Final dilution of the agent						l.d.$_{50}$ titer	Neut. index
	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}		
FW agent alone	5/5**	5/5	5/5	5/5	3/5	1/5	6.3	.....
FW serum 1*	5/5	5/5	5/5	5/5	5/5	0/5	6.5	0
FW serum 2*	5/5	5/5	5/5	5/5	3/5	2/5	6.4	0
FW serum 3*	3/5	3/5	3/5	2/5	2/5	1/5	4.3	100
FW serum 4*	2/5	0/5	0/5	0/5	0/5	0/5	2.2	10,000
Anti VS bovine serum*								
New Jersey type	3/5	1/5	1/5	0/5	0/5	0/5	2.4	7,925
Normal bovine serum*	.....	.....	.....	5/5	4/5	1/5	6.5	0

* With FW agent added.
** Numerator—number of embryo deaths; denominator—number of embryos inoculated.

four samples of FW serum taken on July 15 (sample 1), July 23 (sample 2), August 6 (sample 3), and October 11 (sample 4), a known positive antivesicular stomatitis bovine serum of the New Jersey type and a normal bovine serum. To each of a series of tubes, 0.6 ml. of each of the respective serums inactivated at 56 C. for thirty minutes was added to the series of tubes containing the virus dilutions. After incubation for one hour at room temperature, the mixtures were inoculated in 0.2-ml. volumes into the allantoic cavity of embryonating chicken eggs (table 2).

A similar neutralization test was performed using guinea pigs. Test components were the FW agent, antivesicular stomatitis bovine serum of the New Jersey type and normal bovine serum. To a series of tubes were added 0.5 ml. of a 1.5 dilution of the respective serums and an equal volume of of the agent serially diluted in tenfold increments. The mixtures were incubated at 4 C. for one hour. Two guinea pigs were then inoculated in each rear plantar pad with 0.1 ml. of each mixture. Sufficient FW serum was not available for inclusion in this test. The results appear in table 3, recording the presence or absence of lesions.

TABLE 3—Neutralization Test of the FW Agent in Guinea Pigs

Inoculum	Final dilution of the virus				
	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
FW agent control	ND	++	++	++	± ±
Anti VS bovine serum*					
New Jersey type	++	+ ±	− −	− −	ND
Normal bovine serum	ND	ND	++	++	ND

* With FW agent added; ND = not done; ++ = lesions in both pads of both guinea pigs; ± = questionable lesions; − − = no lesions.

Complement-Fixation Experiments.—Tests were performed with the FW serum and the FW agent. All serums were inactivated at 56 C. for thirty minutes. The antigens of the New Jersey and Indiana types of VS virus were 1:10 suspensions of infected chorioallantoic membranes in tryptose phosphate broth. Three units of complement were used in the test. The components of the antigenic system were incubated in a water bath at 37 C. for twenty minutes and then sheep cells sensitized with rabbit amboceptor were added, followed by an additional incubation period of thirty minutes. Each tube contained 0.25 ml. of antigen, 0.25 ml. of FW serum, 0.25 ml. of complement, and 0.5 ml. of sensitized sheep erythrocytes. Suitable controls of complement, serum, antigen, red cells, and sensitized red cells were included in all tests.

In a complement-fixation test performed with chorioallantoic membrane infected with New Jersey and Indiana VS viruses as antigen in constant strength, and FW serum samples 1, 2, 3, and 4 serially diluted, only sample 4 fixed complement (table 4). Serum taken from a steer hyperimmunized against the FW agent showed complement-fixing antibodies in an identical test.

When various lots of FW-infected embryo were used as serially diluted antigens against New Jersey and Indiana types of hyperimmune guinea pig serum in a complement-fixation test, the antigen fixed complement in the presence of New Jersey

TABLE 4—Complement-Fixation Test for Detection of Vesicular Stomatitis Virus Antibodies in FW Serum Samples

Serum sample (No.)	Date collected	1:10 dilution CA membrane antigen	Serum dilutions					Serum control
			Undil.	1:2	1:4	1:8	1:16	
			Degree of inhibition of hemolysis*					
1	July 15	New Jersey	—	—	—	—	—	—
		Indiana	—	—	—	—	—	—
2	July 23	New Jersey	—	—	—	—	—	—
		Indiana	—	—	—	—	—	—
3	August 6	New Jersey	—	—	—	—	—	—
		Indiana	—	—	—	—	—	—
4	October 11	New Jersey	4	4	4	4	—	—
		Indiana	—	—	—	—	—	—

* Complete (4), lesser degrees (3, 2, and 1), none (—).

TABLE 5—Complement-Fixation Test for Identification of Vesicular Stomatitis Virus Obtained from Third Passage of FW Agent in Embryonating Chicken Eggs

FW agent lot number	Type of guinea pig hyperimmune serum 1:5	Dilution of FW antigen						FW agent control
		Undil.	1:2	1:4	1:8	1:16	1:32	
		Degree of inhibition of hemolysis*						
2	New Jersey	4	4	3	2	1	—	—
	Indiana	—	—	—	—	—	—	
3	New Jersey	4	4	3	1	—	—	—
	Indiana	—	—	—	—	—	—	
4	New Jersey	4	4	4	3	—	—	—
	Indiana	—	—	—	—	—	—	

* Complete (4), lesser degrees (3, 2, and 1), none (—).

antiserum. The FW embryo antigen against Indiana antiserum showed no fixation of complement with any dilution of the antigens (table 5).

Normal guinea pigs infected with FW agent for titration purposes were bled four weeks after inoculation. The serums were used in a complement-fixation test with known New Jersey chorioallantoic membrane antigen in constant quantities. The serums fixed complement in a dilution of 1.40 to 1.80 (table 6).

Discussion

The infection contracted by FW occurred at a time when the New Jersey type of VS virus was the only agent in use in a newly constructed laboratory. Past experience in other laboratories has shown the dangers associated with infectious materials inhaled or deposited accidently in the eyes or nasal passages. The shortness of the incubation period further indicated the magnitude of the exposure or susceptibility of the worker or both. It would appear that the virus occurred in the blood within twenty-four to forty-eight hours after exposure and at the time of the first appearance of the symptoms. In the concentration used for preparation of inoculums, streptomycin and penicillin seemed to have no deleterious effect on the agent.

The FW serum sample 3, taken two weeks after infection, neutralized 100 $l.d._{50}$ egg infective units of virus which in comparison with other virus infections might be considered diagnostic. The fourth sample, collected approximately eighty days after infection, neutralized 10,000 $i.d._{50}$ egg infective units and showed a complement-fixing antibody titer of 1:8. A neutralization test performed in guinea pig foot pads gave similar significant information, considering that the guinea pig is much more resistant to VS virus than the mouse or the egg.

Serologically, the agent seems to have been identified as the New Jersey type of VS virus, although other possible infectious agents were not included in the immunological screening since none was available.

Summary

A scientist, FW, became ill following accidental contact of his face and eyes with vesicular stomatitis (VS) virus, New Jersey type. An agent was isolated by inoculation of the patient's blood into embryonating chicken eggs. The following results show this agent to be New Jersey type VS virus.

1) Neutralization of the FW agent in inoculated eggs was demonstrated with two successive samples of FW serum taken fourteen and eighty days after infection and antivesicular stomatitis bovine serum

TABLE 6—Complement-Fixation Test for Detection of New Jersey Type of Vesicular Stomatitis Antibodies in Serums from 6 Guinea Pigs Inoculated with FW Agents

Guinea pig (No.)	Dilutions of FW agent inoculated	Serum dilutions						Serum control
		1:10	1:20	1:40	1:80	1:160	1:320	
		Degree of inhibition of hemolysis*						
1	10^{-4}	4	4	4	4	—	—	—
2	10^{-4}	4	4	4	—	—	—	—
3	10^{-3}	4	4	4	4	2	—	—
4	10^{-3}	4	4	4	—	—	—	—
5	10^{-3}	4	4	2	—	—	—	—
6	10^{-3}	4	4	4	4			

* Complete (4), lesser degrees (3, 2, and 1), none (—).

of the New Jersey type but not with normal bovine serum or two earlier samples of FW serum taken eight days before or one day after infection.

2) Tests conducted in guinea pigs demonstrated neutralization of the FW agent by antivesicular stomatitis bovine serum of the New Jersey type but not with normal bovine serum.

3) Complement-fixation tests performed with the FW agent as antigen in the presence of New Jersey and Indiana types of VS hyperimmune guinea pig serum and similar tests performed with the serum from FW convalescent guinea pigs in the presence of New Jersey and Indiana type viruses as antigens were positive for the New Jersey type only.

4) Complement-fixation tests performed with FW serum in the presence of New Jersey and Indiana types of VS antigen derived from chorioallantoic membranes of infected embryonating chicken eggs were positive for the New Jersey type only.

Although there is in the literature convincing clinical and serological evidence of infections in man due to VS virus, the present report constitutes the first definite evidence of VS viremia in man.

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Control of Avian Encephalomyelitis by Vaccination

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AVIAN encephalomyelitis (AE) in the fowl is a disease of primary concern to breeders and hatcherymen because it can be transmitted to chicks through the eggs[4,5] and the principal effects of the disease are usually apparent only during the first few weeks after hatching. Among older chickens, its presence will usually not be detected except when flocks of breeding hens are supplying eggs for hatching.

The history of AE since it was first described by Dr. E. Elizabeth Jones[3] has been reviewed by Olitsky and Van Roekel,[6] and by Feibel *et al.*[2] and need not be repeated here except to emphasize that, typically, the disease has appeared in infrequent outbreaks of short duration which pass without being recognized in flocks of breeding hens until signs appear in their progeny. The irregular occurrence of AE and the fact that parent stock remains asymptomatic have made study of this disease difficult. Because of conflicting evidence from field outbreaks and the lack of adequate experimental data, it has sometimes been assumed that the same hens can have AE repeatedly.

It is the purpose of this paper to describe the observations of an outbreak of AE and its sequelae over a period of five years, which suggest (1) that survivors of an outbreak of AE develop an immunity to the disease and should not be discarded as breeders but should be retained as the most certain source of chicks to be hatched free of the disease and (2) that vaccination of prospective breeding stock with the virus of AE during the growing period induces a similar immunity and is an effective method for preventing breeding stock from producing chicks infected with AE.

HISTORY OF OUTBREAKS

This study includes information obtained from the breeding flocks and chicks of a poultry breeding enterprise that produces more than 10 million White Leghorn chicks annually.

The first introduction of outside stock after 1934 was made in the spring of 1941, when hatching eggs were imported from New England.

The first diagnosed outbreak of AE in this flock occurred early in 1942 when losses among chicks were heavy and many adjustments with customers were made.

Another outbreak of AE occurred in December, 1949, among the chicks from one of the two breeding flocks which furnished hatching eggs to a single hatchery.

For convenience, we will designate these breeding flocks as flock A and flock B. The hatchery and flock B were located on the same farm. Flock A was about 180 miles distant and had no physical contact with flock B except that all replacement stock was reared from chicks that were sent from flock B to flock A and that eggs were trucked from A to the hatchery at B twice each week. Separate groups of egg cases were used for the eggs from each flock. Flock A was kept in 92 pens 18 ft. by 40 ft. in size in 18 different houses. First-year pullets in flock A, hatched in 1949, were kept in houses which did not contain older hens.

In December, 1949, a diagnosis of AE among customers' chicks from flock A was first made by the laboratory of the California Department of Agriculture at San Gabriel on the basis of brain inoculation tests. Subsequently, other State laboratories and the laboratory of the San Diego County Livestock Department confirmed the diagnosis on the basis of the histopathology of chicks submitted by other customers. All of these customers had received chicks produced by flock A. The disease followed its usual course and disappeared from among the chicks produced by this flock in two to three weeks.

Analysis of the hatchery records revealed that certain groups of breeding hens in flock A had produced few if any chicks with AE. Although eggs from both hens and pullets were set in the same incubators (Robbins, model 32H) and chicks from both groups were hatched in the same compartments, reports of AE came only from those customers who received chicks from pullets in their first year of egg production. The hatchery records showed that the disease traveled rapidly from house to house among the pullets but failed to appear among chicks in houses containing older breeding hens.

Throughout the time that chicks infected with AE were being hatched from pullets in flock A, chicks from pullets and hens in flock B were frequently hatched in the same compartments of the incubators. No reports of chicks infected with AE

From Kimber Farms, Inc., Niles, Calif.

were received from customers who had obtained these flock B chicks.

During the spring prior to this outbreak, more than 6,000 pullets in flock B were brooded in buildings about 300 feet from the nearest adult birds. After the brooding period, these pullets were moved to new laying houses still farther away from the old hens.

Six weeks after the clinical form of AE had subsided among the chicks produced by flock A, it appeared among the chicks produced by the pullets of flock B. During February of 1950 the chicks hatched from these pullets suffered heavy losses from AE, while the old hens of flock B continued to produce chicks without any important signs of the disease.

Individually pedigreed chicks from flock B were also hatching during February and provided an opportunity to ascertain the accuracy of the observation that the occurrence of AE was limited to chicks from pullets hatched in 1949. The pedigree matings had been housed in buildings near the old hens during the previous November, more than two months before the first occurrence of AE among chicks from this flock. These matings included old males and cockerels mated with old hens, pullets, or both. There were hens and pullets mixed together in some of the pedigree pens. Their chicks were hatched at the same time in the same incubator and they were mixed together in the brooders.

When signs of AE began to appear in the pedigreed chicks the wing band numbers of those that were affected were recorded. Some error is assumed to occur when only clinical signs are used as a basis for diagnosis because occasional chicks may show weakness and unsteady gait for other reasons. Complete absence of signs, therefore, could not be expected even among the progeny of immune hens. The important consideration is the difference in the number of chicks with signs of AE observed among the progeny of pullets and of hens. The total incidence of clinical cases (graph 1) during a three-day period at the peak of the outbreak was about ten times greater among the progeny of pullets than among the progeny of hens that were one or more years older. The age of the sire was unimportant.

These results demonstrate that although some of the hens were exposed to AE by being in pens with the affected pullets, they failed to transmit it to their progeny as did the pullets.

Progeny of Ataxic Survivors.—In order to investigate the possibility that survivors of this outbreak might produce chicks infected with AE, ataxic chicks were removed to separate brooders to be reared with special care. Of this group, 51 pullets and 20 cockerels survived to maturity. These were mated and their eggs were set twice weekly over a period of two months. If some of these ataxic survivors were carriers of the AE virus, one would expect some of their chicks to show signs of having that disease. However, among the 375 chicks hatched and brooded to 3 weeks of age, none exhibited signs of AE.

VACCINATION

The analysis of the 1949–1950 outbreak of AE described indicated that the old hens, in some manner, were protecting their chicks from the disease.

Because it seemed likely that this protection was due to immunity acquired as a result of previous unrecognized exposure, it was decided to study the value of deliberately exposing young prospective breeding stock to the virus of AE before they reached maturity. For this purpose, the virus was propagated, in February, 1950, from the brains of chicks showing active signs of the disease. It was transmitted by intracerebral inoculation in young chicks at approximately monthly intervals. Based on the production of signs of AE in chicks inoculated intracerebrally with 1/20 ml. of dilutions of brain-saline solution mixture, the minimum infective titer of the virus was usually about $10^{-5.0}$.

Method of Exposure.—The virus used for exposure consisted of a 5 per cent suspension of infected chicks' brains in either saline solution or nutrient broth. The first exposure was of a prospective breeding flock, in October, 1950, consisting of pullets hatched during the previous spring. The method of exposure was a wing web stick with a two-pronged vaccinating needle, which was dipped into the brain suspension for each inoculation. About 2 per cent of the pullets in each pen were exposed in this manner.

In 1951 and again in 1952, the AE virus was administered in the same way except that all chicks including males received it when they reached 18 to 20 weeks of age. It was also given to all older chickens at the time they were forced to molt after completing a year's production.

In 1953 and in 1954, the virus was again stabbed into the wing web of all young birds at about 16 to 18 weeks of age, and old birds at molting time, but during these years the AE virus was combined with vaccines for Newcastle disease and fowlpox and was suspended in buffered glycerol.

Effect of AE Virus on Vaccinated Chickens.—Occasionally, a bird which has received AE virus in the wing web for the first time has developed signs of the disease. The incubation period in such cases usually has been from two to four weeks. The number of young chickens developing clinical signs following wing-web vaccination has varied in different broods from none to about 6 per cent, the over-all average among more than 400,000 chickens exposed in this manner being estimated at less than 1 per cent.

In some instances, the exposure of groups of pullets to the AE virus by wing-web stick has been delayed beyond 20 weeks of age and a small number of these have developed clinical signs of AE after an incubation period of four to six

weeks. In three of three attempts, the virus was re-isolated from the brain of such pullets by chick inoculation.

All chicks described in this report as exposed to AE by wing-web vaccination had been vaccinated for Newcastle disease at least once, several weeks previously, in the wing web. In the instances in which AE virus was recovered from the brains of wing-web vaccinated chicks which developed clinical signs, concurrent tests for the presence of Newcastle disease virus were also made by the inoculation of embryos with negative results.

Since the inception of the vaccination program only one diagnosis of AE has been reported to us by a California laboratory. This diagnosis was based on clinical signs and on histopathology. The morbidity from AE can be assumed to have been quite low, since mortality from all causes during the brooding period did not exceed the 4 per cent of extra chicks which this company routinely includes in all deliveries. The occasional occurrence of AE under the conditions described may be reasonably explained by the assumptions (1) that occasional hens may be "healthy carriers" of the disease, (2) that a few birds fail to contract the disease at the time of vaccination but do get it later on, and (3) that active immunity to AE is relatively but not absolutely permanent, so that a few birds become susceptible in later life and contract the disease a second time.

Since the outbreak of AE in 1949–1950 there has been no new outbreak of the disease under conditions where:

1) the survivors of AE have been retained as breeders,

2) the live virus of AE has been systematically distributed to the birds,

3) active infections with AE have been known to occur among a few wing-web inoculated birds as demonstrated by the development of signs of AE and the recovery of AE virus, and

4) eggs have been hatched every week of the year so there could have been no important recurrence of AE infection in the breeding flocks without its being detected.

Challenge Tests.—Two experiments were conducted in which young White Leghorn chicks were inoculated in the wing web by the stick method with approximately 0.01 ml. of a 20 per cent suspension of a pool of AE-infected chick brains in pH 7.2 buffered saline. The 50 per cent end point (c.l.d.*$_{50}$) of this virus pool was calculated according to the method of Reed and Muench[7] as described by Van Rooyen and Rhodes.[10] In two separate titrations four months apart, this pool of virus was found to have a c.l.d.$_{50}$ titer of 1:632 and 1:682 when administered intracerebrally to 12-day-old and 10-day-old chicks, respectively, in a dose of 0.05 ml. From these titrations the wing-web exposure dose was calculated to contain approximately 25 c.l.d.$_{50}$ doses of virus.

This same pool of AE virus (stored at −20 C.) was later used to challenge the chicks in both experiments. The challenge dose was administered intracerebrally and consisted of 0.2 ml. of a 10 per cent suspension of the virus pool, calculated to contain approximately 250 c.l.d.$_{50}$ doses of virus or ten times the initial wing-web dose. Dihydrostreptomycin was added to the challenge material at the rate of 5 mg. per milliliter.

The control chicks in each experiment were brooded in the same room as the wing-web inoculated chicks but in different batteries. No special effort was made by the caretaker to avoid carrying infection from one group to another. After administering the challenge dose, the inoculated and control chicks of each experiment were placed in the same pen.

In experiment 1, 59 chicks were inoculated in the wing web at 3 days of age. Of these, 4 developed clinical signs after incubation periods ranging from nineteen to twenty-eight days. In experiment 2, 62 chicks were inoculated in the wing web at 14 days of age; 2 showed signs of AE after forty to fifty-two days.

The chicks in both groups were challenged intracerebrally on the same day, when those in experiment 1 were 84 days of age and those in experiment 2 were 66 days of age. A few chicks in each group died within forty-eight hours following the intracerebral inoculation. These losses were attributed to trauma and were excluded from the summarized data.

In each experiment following challenge inoculation, the incubation period to the first clinical signs was twelve days for the controls and fourteen days for the wing-web inoculated groups. Most of the morbidity in all groups occurred by the twentieth day after challenge and the experiments were terminated the twenty-eighth day.

The total mortality (graph 2) from AE in experiment 1, where the chicks were inoculated in the wing web at 3 days of age and challenged eighty-one days later, was 17 among 49 (34.7%) for the wing-web inoculated chicks; 40 among 46 (87 %) for the controls. In experiment 2, where the chicks were 14 days old when they were inoculated in the wing web and were challenged fifty-two days later, the mortality was 24 of 57 (42.1 %) for the wing-web inoculated chicks; 39 of 44 (88.6 %) for the controls.

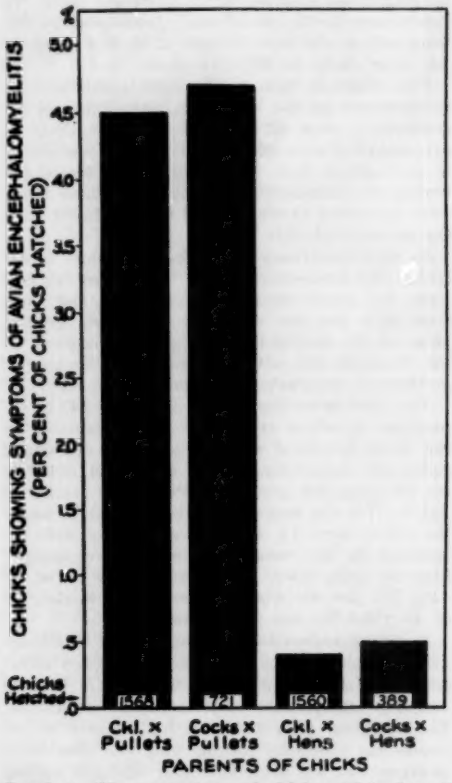
It seems reasonable to assume that challenge with 250 c.l.d.$_{50}$ doses of virus administered intracerebrally is much more severe exposure than is normally experienced under natural conditions. The fact that even with this high degree of exposure the mortality among wing-web inoculated chickens was less than half that observed among the controls clearly supports the circumstantial evidence, derived from five years of planned flock exposure and vaccination, that a useful degree of

* Chick-lethal-dose$_{50}$ is that dilution of virus which, in the dosage stated and by the route indicated, induced the fatal disease in 50 per cent of the chicks inoculated.

immunity develops following wing-web inoculation with the virus of AE.

DISCUSSION

Transmission Through the Egg.—It has been demonstrated in laboratory experiments[5,8] that by inoculating fertile eggs and embryos with the virus of AE, chicks can be produced which show signs of the disease within a few hours after hatching. Since the incubation period in the artificially induced disease which follows intracerebral inoculation is rarely less than nine days, it seems reasonable to conclude that the heavy mortality observed in natural outbreaks among chicks during the first 10 days after hatching is a result of the transmission of virus from a hen to her progeny through the egg.



Graph 1—The percentage of chicks which showed clinical signs of avian encephalomyelitis (weakness, unsteady gait or tremor) among chicks during three days at the peak of an outbreak. The chicks (ckl = cockerel) ranged from 1 to 3 weeks of age.

In the 1949–1950 outbreak reported here, some clinical signs were observed at the time chicks were removed from the incubators; many more were observed within twenty-four hours and morbidity in some broods was as high as 50 per cent. Nearly all of it occurred, however, during the first week or ten days after hatching. The absence or low incidence of the disease among chicks more than two weeks of age provides additional evidence that most of the mortality resulted from egg-borne virus.

This experience is not universal, however, and some investigators have reported high mortality at later ages. Completely satisfactory explanations of such cases should not be expected because of the complex interrelationships that develop in the field among different strains of chickens, different diseases that produce similar signs, different strains of the same infectious agent, fragmentary history of the parent flocks, and the unavoidable errors of diagnosis.

In actual practice, the present uncertainty concerning transmission of the disease after hatching need be of little importance. When transmission of the disease through the egg is prevented by resorting to breeding from immune parents, there is little reason to expect that environmental exposure, under reasonably clean conditions of brooding, will cause serious mortality.

Immunity of Survivors.—If any of the survivors of an outbreak of AE were to be capable of infecting their subsequent progeny, we might assume that it would be those which lived to maturity in spite of a specific clinical attack.

Investigations of AE by Jones[4] and by Bottorf[1] provided evidence that survivors of this disease are unlikely to be "carriers" capable of transmitting the virus to their progeny. These investigators reported the results of a study in which an effort was made to hatch additional AE-infected chicks from parents which had previously produced infected chicks. Among the progeny from five hatches no AE was diagnosed. Cockerels and pullets which survived the original outbreak were also mated but their chicks from five hatches likewise gave no evidence of AE. Van Roekel and co-workers,[8] reported that no positive evidence of AE was detected

among 696 chicks hatched over a three-month period from eggs laid by 26 ataxic pullets. These pullets had survived an attack of AE and came from seven different flocks. A second hatching trial conducted several months later with part of the birds used in the first trial also failed to produce infected chicks. A similar test, conducted by the present authors with survivors of the outbreak described at the beginning of this paper, also produced negative results.

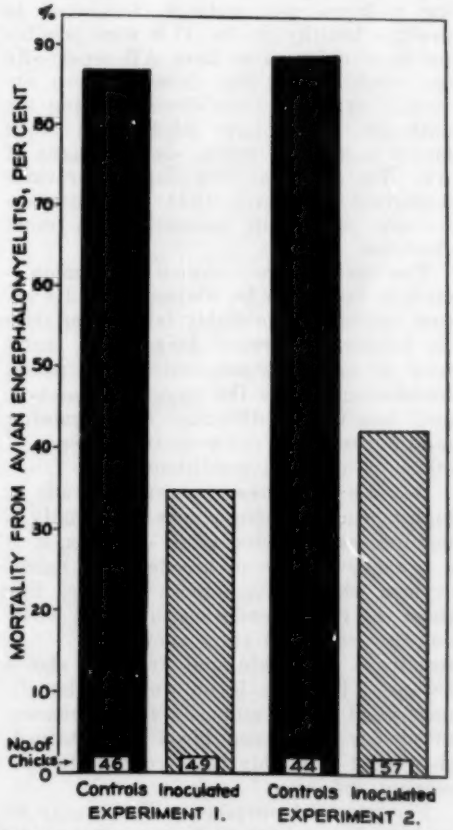
That artificial exposure to the AE virus might actually provide protection against "challenge" by intracerebral inoculation with the virus was apparently first recognized by Jungherr and Minard [5] in 1942. They reported that of eight different non-cerebral routes tried, the chicks inoculated in the peritoneal cavity were not susceptible to intracerebral re-inoculation. The quantities of virus used in the inoculations were not stated. It would appear, therefore, that from the earliest studies of this disease we have evidence to suggest that it can be controlled by a program of judicious exposure of prospective breeding stock to the live virus.

There is no agreement among investigators, however, that such a program for control would be either desirable or effective and the generally recommended procedures are very different. Although Jungherr and Minard [5] recognized that "The serum of convalescent chicks possesses weak specific neutralizing antibodies," they concluded that "No control measures are available. Visibly affected chicks should be culled, but condemnation of affected parent stocks should be contemplated only after repeated outbreaks." One of the 12 flocks observed by them showed evidence of such repeated outbreaks.

Seven years later, Van Roekel *et al.*[9] suggested that "Affected flocks should be given the best of care and survivors of an outbreak in all probability should not be used for breeders."

In 1952, Olitsky and Van Roekel [6] reported in their review that "Van Roekel and Jungherr and co-workers offer little encouragement at the present time for the control of the disease. They believe that infected birds should be culled and disposed of. They advise good husbandry practice and hygiene and condemn any restocking of older, infected flocks with day-old chicks. When breeders are saddled with epidemics arising in repeated waves, relief from this burden, then, is to clear out the stock and start anew."

Such recommendations to avoid the use of survivors for breeding arise from the conflicting evidence concerning the possibility of repeated outbreaks of AE in the same hens. One of the most baffling characteristics of AE has been its erratic occurrence. As indicated above, it has been reported that normal hatches have been preceded and followed by infected ones from the same flock, suggesting that the hens develop little useful immunity and contract the disease repeatedly. No evidence that this occurs has been found in



Graph 2—The comparative incidence of avian encephalomyelitis in control and wing-web inoculated chicks following intracerebral challenge with ten times the quantity of virus used in wing-web inoculation.

the flocks of the present study during more than five years of observation.

Because the erratic appearance of AE has contributed to the beliefs (1) that "healthy carriers" exist to spread the disease, and (2) that hens may be expected to have AE more than once, it is especially important that any reports of repeated outbreaks of AE in the same hens be carefully studied and reviewed.

It is possible that a hatchery receiving eggs from several supply flocks may have repeated outbreaks of AE, as was reported here when the disease appeared in one flock and then, nearly two months later, in the other. However, the chicks from old hens, whether in separate houses or mixed with affected pullets in the same or adjoining pedigree pen matings, continued to produce healthy chicks. If it were possible for most chickens to have AE repeatedly one would expect that these old hens, obviously exposed to the disease during the outbreak, would have produced a much larger number of chicks showing signs of AE. The fact that they did not provides important evidence that this disease induces permanent immunity in most chickens.

Practical Control.—Avian encephalomyelitis is known to be widespread.[2] Its actual incidence is probably far greater than its known occurrence because its usual form in adults is sub-clinical and passes undetected unless the eggs are hatched, and because of difficulty in diagnosing mild outbreaks in chicks in the presence of other pathological conditions.

Because this disease sometimes leads to high mortality among chicks but usually is not noticeable among adult chickens, it is a primary concern of breeders and hatcherymen who produce chicks for sale. For them the recommendation to ". . . clear out the stock and start anew . . ."[6] is usually a desperate and fruitless choice because (1) there is no way to identify unexposed flocks and (2) if, by chance, previously unexposed birds are obtained, they must inevitably be susceptible to subsequent infection.

For practical purposes a disease may be said to be under satisfactory control if, over a period of years during which the causative agent is known to be present and active, clinical manifestations of the disease are reduced to very low levels or eliminated entirely.

During recent years such control has been achieved for two other widespread virus diseases, Newcastle disease and infectious bronchitis, by the vaccination of immature birds with selected strains of the virus involved. Our five years of observation indicates that this disease also can be controlled effectively in the same manner.

SUMMARY

During an outbreak of avian encephalomyelitis (AE) in 1949–1950, it was observed that high mortality occurred only among the progeny of the youngest generation of breeding hens. This suggested that the older hens had had the disease previously, had developed immunity to it and, therefore, were incapable of transmitting AE through their eggs to the chicks.

Since that time, there has been no serious recurrence of the disease among baby chicks under conditions where:

1) the survivors of the 1949–1950 outbreak of AE have been retained as breeders,

2) the live virus of AE has been systematically distributed to young chickens before they reach maturity and to older ones at the time of their annual molt,

3) the disease is known to be present in active form as shown by the demonstration of clinical signs by a few of the inoculated chickens and by recovery of AE virus from them, and

4) eggs have been hatched every week of each year so that no important recurrence of AE could have passed unnoticed.

In two different experiments, young chicks inoculated in the wing web with 25 chicken lethal dose$_{50}$ (c.l.d.) quantities of AE virus later suffered less than half the mortality observed among controls following intracerebral challenge with 250 c.l.d.$_{50}$ quantities of the virus.

These results indicate that AE can be satisfactorily controlled by the vaccination of immature chickens with the live virus which causes the disease, thereby enabling them to develop a useful degree of immunity to the disease before their eggs are used for hatching.

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Laboratory Techniques for Isolation and Propagation of Vibrio from Cattle

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IN THE study of bovine vibriosis, isolation of the infective agent from the reproductive tract has been the most reliable method for diagnosing the disease.

The purpose of this report is to describe the laboratory techniques used for the isolation and propagation of Vibrio from the vagina, uterus, placenta, and aborted fetuses of cows, the prepuce and semen of bulls, and the genital organs of both at the time of necropsy.

Numerous techniques for the isolation of Vibrio have been used.[2, 6-14] On the basis of biochemical tests, two types of Vibrio have been identified by cultural methods.[2]

The culture medium found most satisfactory for primary isolation of Vibrio was blood agar in Petri plates similar to that used by Terpstra and Eisma.[13] The medium consisted of 15 Gm. of agar, 10 Gm. of peptone, 5 Gm. of sodium chloride, and either 5 Gm. of commercial beef extract in 1 liter of distilled water or beef infusion to make 1,000 ml. After autoclaving and cooling the nutrient agar to about 52 C., 10 per cent of defibrinated bovine blood was added before pouring the plates. An added stimulus for Vibrio growth was obtained by incorporating 0.3 per cent thiol* in the above medium. The final pH was 7 to 7.2. The freshly prepared blood agar plates were incubated at 37.5 C. for twenty-four to forty-eight hours before use to reduce excess moisture and to check for contamination. Thereafter, they were stored at 4–7 C.

Thiol[7] or thioglycolate[8] medium was used for culturing materials free of extraneous bacteria and for propagating stock cultures.

For the isolation of Vibrio from cows, vaginal mucus and uterine swabs were collected with a special instrument.[4] From 0.1 to 0.5 cc. of vaginal mucus was inoculated onto each of two or three blood agar plates per cow. The mucus was dispersed over the surface of the medium with a wire spreader. The uterine swabs were either spread directly onto plates or washed in nutrient broth for later inoculation. In culturing these materials, Vibrio was isolated most frequently from mucus obtained at the cervico-vaginal juncture.[5]

Semen was collected in the artificial vagina and cultured by depositing 1 drop onto a blood agar plate from which it was spread by serial dilution onto three additional plates. Two or three such series of plates improved the possibility of isolating Vibrio. Preputial samples were obtained by using either the apparatus mentioned above for sampling cows[4] or the one described by Bartlett[1] for sample collection in *Trichomonas foetus* examination. These samples were washed from the instrument into about 6 cc. of saline and either cultured directly or diluted with an equal volume of broth. One drop of either of the above samples was deposited onto the surface of a blood agar plate from which it was spread by serial dilution onto three additional plates. Four such series, making a total of 16 plates, were used for each preputial sample. In most instances, samples from the prepuce contained numerous species of bacteria which interfered with the isolation of Vibrio. The isolation of Vibrio by culturing semen has been more satisfactory than by culturing preputial samples.[5]

The contents of the rumen and abomasum of aborted fetuses were examined routinely both microscopically and culturally for Vibrio. Blood agar and thiol cultures were made of the liver, spleen, lungs, kidneys, intestines, stomach contents, and fetal placenta. Culturing the stomach contents was the most satisfactory method for isolating Vibrio from aborted fetuses.

The reproductive organs from cows and bulls slaughtered for reason of infertility were cultured on blood agar and thiol. Samples for culture were taken from the vagina posterior to the cervix, cervico-vaginal juncture, mid-cervix, uterus, Fallopian tubes, bladder, and urethra from cows, and from the testes, epididymis, vas deferens, ampulla, seminal vesicles, bladder, urethra, and fornix of bulls. Vibrio were isolated only from the uterus and cervico-vaginal juncture of cows and from the prepuce of bulls after slaughter.[5]

The inoculated cultures were placed in desiccation jars, which were then evacuated to a pressure of approximately 43 cm. of mercury after which 10 per cent carbon dioxide by volume was added, and incubated at 37 C. for three to five days.

The incubated plates were first examined with a stereoscopic microscope of 13X magnification. Two forms of Vibrio growth were commonly found: one, a distinct colony formation, and the other, a continuous spreading growth. The colonies were circular, varying in size from punctiform to 3 mm. in diameter, with an average size of

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* Bacto-thiol (B307), Difco Laboratories, Philadelphia, Pa.

about 2 mm. The larger colonies were entire, convex, and contained granular striations. The continuous spreading form of growth occupied areas from 1 ml. in diameter to coverage of the entire plate. The pathogenic and nonpathogenic types of Vibrio mentioned earlier could usually be identified by the color and physical qualities of the growth on blood agar. The pathogenic type, or *Vibrio fetus*, produced white colonies or spreading growth with a bluish hue at the periphery. The nonpathogenic type generally produced brownish green spreading growth with a metallic sheen and occasionally partial hemolysis. However, in a few instances the two types could not be differentiated except by biochemical methods.[2]

All bacterial growth resembling the two types described were examined under oil immersion, preferably with a phase contrast microscope. However, a satisfactory staining technique was developed for use with the standard microscope in which motile bacteria remained active for several minutes. The stain consisted of crystal violet* 0.1 Gm., methylene blue chloride** 0.5 Gm., ammonium oxalate† 0.2 Gm., ethyl alcohol (95 %) 5.0 ml., and 120 ml. of distilled water. After dissolving the dye in alcohol and distilled water, 187 ml. of physiological saline was added. A loopful of the stain was placed on a microscope slide to which Vibrio were added with a platinum loop or needle. A cover slip was applied for oil immersion examination. Vibrio as well as other bacteria gradually took up the stain and were plainly visible with the standard microscope.

For pure culture collection Vibrio were transferred from the original medium to thiol[7] and incubated in an atmosphere of 10 per cent carbon dioxide at 37 C. for three days. Cultures with insufficient growth were transferred to fresh thiol at weekly intervals until adapted to artificial mediums. The stock culture collection has been kept in a dark closet at room temperature and maintained by transferring at monthly intervals.

* Crystal violet dye content 91 per cent C.I. No. 681, National Aniline Division Allied Chemical & Dye Corp., New York 6, N. Y.

** Methylene blue dye content 88 per cent C.I. No. 922, National Aniline & Chemical Co., Inc., New York 6, N. Y.

† Ammonium oxalate code 1307, General Chemical Division Allied Chemical & Dye Corp., New York, N. Y.

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A Suitable Basal Medium for Evaluation of the Fermentative Capacity of *Erysipelothrix Rhusiopathiae*

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Literature Cited

The literature attests the wide divergence of opinion which exists relative to the fermentative capacity of *Erysipelothrix rhusiopathiae*.[1-3, 5-7, 9-13, 15, 19-21]

A number of different basal mediums have been described and a variety of results have been obtained through the use of these mediums.[2, 4, 5, 7, 11, 12, 20] This factor alone indicates the need for an acceptable basal medium which can serve as a basis for the general evaluation of the fermentative capacity of *Ery. rhusiopathiae*.

In a recent publication, Tiffany and Wheaton[12] described a medium for the cultivation of *Ery. rhusiopathiae* which appeared to support the growth of this organism very well. It was felt that with proper modification this medium could be redesigned to serve as a basal medium for the determination of the fermentative faculties of *Ery. rhusiopathiae*.

This paper includes a description of a basal medium, its preparation, partial evaluation, and some observations which were made regarding its performance under test.

Materials and Methods

Twenty strains of *Ery. rhusiopathiae* were obtained from various isolations made in the bacteriological laboratories at Michigan State College, East Lansing. The strains were identified as to the source, animal, bird, or stock culture from which they were obtained according to the following code: P, pig; S, sheep; T, turkey; and L, lyophilized laboratory stock culture.

All the strains were in a smooth phase, P-41, S-18L2, and P-Milan excepted. These latter three appeared to be in the rough, smooth-rough, and rough phases, respectively.

The medium used for the isolation, reconstitution, and maintenance of *Ery. rhusiopathiae* strains prior to and during this work was nutrient-tryptose broth.[18] Since a modification of this medium was to be the basal medium for the fermentation reactions, the following tests were made on individual components for the purpose of ascertaining their capacity to support a fermentation reaction.

The following mediums were prepared:

Medium A—	bacto-peptone	2.0 Gm.
	sodium chloride (NaCl)	0.5 Gm.
	thiamine hydrochloride	0.5 mg.
	distilled water to	100.0 ml.
Medium B—	bacto-tryptose	2.0 Gm.
	NaCl	0.5 mg.
	thiamine hydrochloride	0.5 mg.
	distilled water to	100.0 ml.
Medium C—	bacto-beef extract	2.0 Gm.
	NaCl	0.5 Gm.
	thiamine hydrochloride	0.5 mg.
	distilled water to	100.0 ml.
Medium D—	bacto-peptone	2.0 Gm.
	bacto-tryptose	2.0 Gm.
	bacto-beef extract	2.0 Gm.
	NaCl	0.5 Gm.
	thiamine hydrochloride	0.5 mg.
	distilled water to	100.0 ml.

Bacto-phenol red indicator was added to each of the above mediums in sufficient quantity to yield a final concentration of 0.01 per cent. The reaction was adjusted electronically to pH 7.6 using 0.5 normal sodium hydroxide (NaOH). These mediums were tubed in 10 by 75-mm. pyrex tubes in which gas inserts had been placed. Sterilization was accomplished by autoclaving at 121 C. for 8 minutes. These were then cooled and inoculated from an 18-hour broth culture of *Ery. rhusiopathiae*. The results, which were read after 48 hours of incubation at 37 C., are recorded in table 1.

TABLE 1—Fermentation of the Various Components of the Proposed Basal Fermentation Medium

Medium	24 hours			48 hours		
	Growth	Acid	Gas	Growth	Acid	Gas
A	−	−	−	+	−	−
B	±	−	−	+	−	−
C	−	−	−	±	−	−
D	+	−	−	+	−	−

+ indicates growth, acid or gas production; − indicates no growth, no acid, or gas production.

The basal medium was dissolved by gentle heating. The formula for 1 liter is:

bacto-beef extract	3.0	Gm.
bacto-peptone	5.0	Gm.
bacto-tryptose	20.0	Gm.
NaCl	5.0	Gm.
thiamine hydrochloride	0.005	Gm.

A 0.2 per cent solution of bacto-phenol red indicator was prepared in accordance with the instructions outlined in Mackie and McCartney.[8] Two and five tenths milliliters of this 0.2 per cent stock solution was added to each 100 ml. of basal medium. The reaction was adjusted to pH 7.6.

The carbohydrates (this term is used to include sugars, polyhydric alcohols, etc.) were prepared by

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dissolving 1.5 Gm. of these compounds in 100 ml. of distilled water. One milliliter of these solutions was then added to separate tubes containing 2.0 ml. of the basal medium prior to autoclaving at 121 C. for 8 minutes.

Stock solutions of maltose, fructose, arabinose, dextrin, and xylose were prepared and sterilized by filtration. They were then aseptically added in the desired amounts to the previously sterilized basal medium.

The final concentration of carbohydrate was 0.5 per cent and of indicator, 0.01 per cent.

Inoculation of this medium was made from an 18-hour broth culture of *Ery. rhusiopathiae*. The results were read after 48 hours of incubation at 37 C.

The controls consisted of 2.0 ml. of the basal medium containing the indicator to which was added 1.0 ml. of sterile 0.85 per cent saline.

The results (table 2) are expressed as 1, 2, 3, 4, or negative depending on a visual comparison with the controls. This classification is based on an estimated degree of color change of the indicator, the control being accepted as (—) or no change, the 4 being a very decided change to the yellow (acid) color.

Results and Discussion

The inconsistency with which different strains of *Ery. rhusiopathiae* attack various carbohydrates[1, 5, 6, 17, 18, 20] has been pointed out. In consideration of the work presented by these authors, one can obtain a spectrum of compounds which are attacked by this organism. Further consideration of their work indicates that numerous different basal mediums were utilized to bring out the active members of this fermentative spectrum and that no one medium alone was sufficient to establish and/or include all the various fermentable compounds within its limits.

Deem and Williams[5] and Tenbroeck[11, 12] found that the addition of serum to the medium brought out the saccharolytic powers of *Ery. rhusiopathiae* to a greater extent than those mediums wanting in this substance. However, they also recognized the potential inherent ability of some serum enzymes to split certain of the carbohydrates, thus giving false positive results. Vera[14] also pointed out the possibility of false positive results arising from fermentable substances in the culture medium other than serum, e.g., meat extracts and peptones. An attempt has been made to avoid or evaluate these variables through the exclusion of serum and a fermentative assay of the basic culture ingredients.

The results (table 2) show an increased spectrum of compounds to be fermented by this organism as well as a consistency of fermentation on the respective substances. These results reaffirm the work of many investigators as to the established ferment-

TABLE 2—Fermentative Activity of Twenty Strains of Erysipelothrix Rhusiopathiae in the Proposed Basal Medium

Strain (No.)	Carbohydrates																
	Glucose	Fructose	Galactose	Lactose	Sucrose	Salacin	Rhamnose	Mannose	Maltose	Dextrin	Xylose	Arabinose	Sorbitol	Inositol	Esculin	Mannitol	Control	Control
P-3	3	3	3	3	—	—	—	1	—	—	4	2	—	—	—	—	—	—
P-33	3	3	3	2	—	—	—	1	—	—	4	2	—	—	—	—	—	—
P-42	4	4	4	3	—	—	—	1	—	—	4	2	—	—	—	—	—	—
P-N	4	4	4	3	—	—	—	1	—	—	4	2	—	—	—	—	—	—
P-1	4	4	4	2	—	—	—	1	—	—	4	2	—	—	—	—	—	—
P-2	4	4	3	3	—	—	—	1	—	—	4	2	—	—	—	—	—	—
T-732	4	4	4	3	—	—	—	1	—	—	4	2	—	—	—	—	—	—
P-32	4	4	4	3	—	—	—	1	—	—	4	2	—	—	—	—	—	—
P-41	2	2	2	2	—	—	—	1	—	—	4	2	—	—	—	—	—	—
S-18L2	3	3	3	2	—	—	—	1	—	—	4	2	—	—	—	—	—	—
P-14549	4	4	3	3	—	—	—	1	—	—	4	2	—	—	—	—	—	—
P-14591	4	2	3	4	—	—	—	1	—	—	4	2	—	—	—	—	—	—
P-W	4	4	4	4	—	—	—	1	—	—	4	2	—	—	—	—	—	—
P-14726	4	4	4	4	—	—	—	1	—	—	4	2	—	—	—	—	—	—
P-14774	4	4	4	4	—	—	—	1	—	—	4	2	—	—	—	—	—	—
P-14777	4	4	4	4	—	—	—	1	—	—	4	2	—	—	—	—	—	—
P-3379	4	4	4	4	—	—	—	1	—	—	4	2	—	—	—	—	—	—
P-MC	4	4	4	4	—	—	—	1	—	—	4	2	—	—	—	—	—	—
P-14924	4	4	4	3	—	—	—	1	—	—	4	2	—	—	—	—	—	—
P-Milan	3	2	2	2	—	—	—	1	—	—	4	2	—	—	—	—	—	—

The numbers indicate varying degrees of acidity without gas production: (1) slight, (2) and (3) intermediate degrees, (4) greatest acid production, (—)growth with no acid or gas produced.
P—pig; S—sheep; T—turkey.

able compounds such as glucose, fructose, lactose, and galactose. These results also represent information of the consistent fermentation of compounds which previously have been of questionable position in this particular fermentative spectrum. The difference in fermentation by strains, such as is indicated by the system of recording of the results in table 2, appears to be in the rate of attack rather than type of compound affected. This rate is more pronounced with the smooth than with the rough strains but some variation can be observed within the group of strains which appear to be smooth.

The work reported herein does not establish whether or not the rate of fermentation is directly related to the rate of growth. However, it is the author's opinion that the rate of growth and fermentation are more or less independent since the rough and smooth strains appear to grow equally well in both the basal and the maintenance mediums.

For a basal medium to be of practical value, it should lend itself to storage. It was found that after storage for six months in a refrigerator no perceptible alteration in this medium could be detected.

It is recognized that 20 strains is an insufficient number on which to base any definite conclusions as to the fermentative abilities of this organism. However, a trend has been established which is felt will be reaffirmed upon future application and evaluation of this basal medium indicating its adaptability to the function for which it was designed.

SUMMARY

A basal medium for the evaluation of the fermentative capacities of *Erysipelothrix rhusiopathiae* has been described. Twenty strains of this organism were tested using this medium and the results recorded. In general, glucose, fructose, lactose, galactose, mannose, xylose, and arabinose were consistently fermented with the production of acid without gas. A difference in rate of fermentation was observed which appeared to be independent of the rate of growth. This was most clearly demonstrated between the rough and the smooth strains, the rough being slower to ferment the test compounds, though of a similar rate of growth as the smooth strains.

Absence of serum, ease of preparation, relatively low cost, high sensitivity, and stability during storage combine with consistency of fermentation and wide spectrum to yield a basal medium which appears to be acceptable for the evaluation of the fermentative activities of *Ery. rhusiopathiae*.

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